2008

Laccase-catalyzed removal of various aromatic compounds from synthetic and refinery wasterwater

Aaron Steevensz
University of Windsor

Laccase-Catalyzed Removal of Various Aromatic Compounds from Synthetic and Refinery Wastewater

By:

Aaron Steevensz

A Dissertation
Submitted to the Faculty of Graduate Studies
Through the Department of Chemistry and Biochemistry
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy at the
University of Windsor

Windsor, Ontario, Canada

2008

Your file Votre référence
ISBN: 978-0-494-47115-9
Our file Notre référence
ISBN: 978-0-494-47115-9

AUTHOR'S DECLARATION OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances to my appendix.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

Laccase SP 504 from *Trametes villosa,* a fungal laccase, was investigated for its capacity to catalyze the oxidative polymerization of hazardous aromatic pollutants (phenol, o-, m-, p-cresol, aniline, o-, m-, p-toluidine) in the presence of O_2. Experiments were conducted to determine the optimum pH, minimum enzyme concentration for $\geq$ 95 % conversion of substrate, minimum PEG concentration for optimum effect (if applicable), and the effect of PEG molecular weight on removal efficiencies. Other factors investigated were: the fate of PEG in the reactor, removal over a 3 h reaction period, activity over 3 h reaction period, and the effect of reducing anions and halides in p-cresol conversion. For the three cresol isomers a preliminary kinetic study was done with and without PEG. Lastly the effectivness of laccase in the removal of phenol in 5 refinery samples was compared to the removal of phenol in synthetic wastewater.

DEDICATION

This Thesis is dedicated to my parents:

Dr. Richard Steevensz and Sandra Steevensz

"Don't let a win go to your head or a loss to your heart..."- Public Enemy

And my many brothers and sister

Jean-Paul, Jason, Michael, Jordan and Ashley

"whatever goes down goes down and that's cool" - slosh

For all of your love and support in all my endeavors in and out of school, I only hope that one day I can return the favour.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Keith E. Taylor and our collaborators from Civil and Environmental Engineering, Drs. Jatinder Bewtra and Nihar Biswas for their support, guidance, and patience through the years. Words can not express how appreciative I am for the opportunity that you have given me. I would also like to thank Drs. Vacratsis and Eichhorn as committee members for their time and suggestions.

I would like to thank my lab mates who have become more than colleagues over the years and I consider them all as friends: Mohammad Mousa, Joey Patapas, Rob Biswas, and **even** the engineers: Katy Modaressi, Beeta Saha, and Riya Dutta. I would like to formally apologize to them for subjecting them to my music in the lab for so many years. I will also miss Betsie.

To my other friends: Alexis, Lacy, Travis, Eric, Brad, Jason, Tanveer, Levi, Kevin, John, thanks for all the pleasant distractions, help, and support through the years. Without such good friends none of this would mean as much.

Thanks!!!!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SCHEMES

Chapter 1: Introduction

1.1 Pollutants

1.1.1 Industry

"Phenols," "Total Phenols," or "Phenolics" are often considered to be interchangeable in wastewater treatment technology referring to phenol or a mixture of phenols in wastewater (Beszedit and Silbert, 1990). Phenols, including o-, m-, and p-cresols can be major organic constituents found in effluents from chemical and allied industries including: petrochemical, oil refinery, chemical and glass manufacturing, steel plants, metal refining, ceramic plants, phenolic resin manufacturing, and textile industries (Veeresh et al., 2005; Saravanan et al., 2008; Juang and Tsai, 2006; Yan et al., 2006; Bai et al., 2007). Phenol is used in disinfectants, pesticides (USEPA/OPP Pesticide code 064001), resins, and as a chemical intermediate for bisphenol A (BPA), nonylphenol, adhesives, pharmaceuticals, and plastic production (Busca et al., 2008). The largest use of phenols (35%) is for phenol-formaldehyde based resins (originally as Bakelite) now widely used as plywood adhesives due to their low cost (Busca et al., 2008). BPA production for epoxy resins accounts for 20% of phenol use while 16% is used for making precursors to nylon 6 and other polyester polyols (Busca et al., 2008). Cresols belong to a class of chemical compounds used extensively in resin manufacturing such as epoxy cresol-novolac resins (o-cresol) (ECN) used as a sealing material for integrated circuits on silicon chips because they are relatively flame retardant with reduced moisture uptake and decreased branching (Kavitha and Palanivelu, 2005; Lin-Gibson et al., 2002). o-Cresol is a chemical intermediate for a number of herbicides and pesticides including: dinitro-o-cresol (DNOC), 4-chloro-2-methylphenoxyacetic acid (MCPA), gamma(4-chloro-2-methylphenoxy)-propionic acid (MCPP), and 4-(4-chloro-2-methylphenoxy)-

butyric acid (MCPB). m-Cresol is approved for use in pesticides (USEPA/OPP Pesticide code: 022102) as well as being an intermediate for thymol, used in cough and cold medicine and for the synthesis of tri-nitro-m-cresol for use in explosives (TOXNET). p-Cresol is used in making antioxidants like 2,6-di-tert-butyl p-cresol (BHT, butylated hydroxytoluene) and Tinuvin 326 which absorbs UV light and is added to polyethylene and polypropylene films to protect materials against photodegradation (TOXNET). Cresols and phenol are also used for: food antioxidants, perfume and fragrance manufacturing, sulphur and chromium dyes, magnet wire coating, metal degreasing agents and as or in disinfectants (TOXNET).

Anilines are found in effluents of the timber industry, printing and publishing, oil shale recovery, oil refineries, chemical and coal conversion plants (Shackelford et. al., 1983, Ellis et al., 1982). Aniline is mainly used for making dyes and printing inks as well as a chemical intermediate for synthesis of explosives, photographic chemicals, isocyanates, pesticides, pharmaceuticals and recently polyanilines (PANI) because of its high chemical and thermal stability and good electric conductivity with a broad range of potential applications (Cruz-Silva et al., 2005; Xing-yu et al., 2007). o-, m-, and p-Toludines are used in the printing and textiles industry as chemical intermediates for dyes, antioxidants for rubber, and vulcanization accelerators (TOXNET).

1.1.2 Health

Phenol and cresols are classified by the USA Environmental Protection Agency (EPA) as persistent, priority, toxic chemicals. Phenol is a class D compound determined not to be carcinogenic to humans whereas the cresols are class C compounds or possible human carcinogens (Singh et al., 2008; TOXNET). All cresol isomers are found to have

genotoxic effects alone or in combination (Thompson et al., 1994). It has been reported that m-cresol is less of an eye and skin irritant than phenol followed by o-cresol and p-cresol being the most (TOXNET). People exposed to these chemicals are mainly workers in the manufacturing sectors of the pre-stated industries as well as lab workers. The main types of exposure are through inhalation as well as dermal or oral uptake. Rapid absorption and severe systemic toxicity can occur through any route of exposure usually due to attack of the central nervous system (CNS), heart, lungs and kidney (TOXNET). Phenol is caustic to tissue, eyes and the respiratory tract (Singh et al., 2008; Busca et al., 2008). The ingestion of 1.0 g of phenol is fatal for humans (Busca et al., 2008).

Nitrogen-containing aromatic compounds (NACs) have been shown to be toxic and or mutagenic (Xing-yu et al., 2007). Aniline, m-,p-toluidines are not proven human carcinogens. o-Toluidine gives rise to DNA adducts and is classified as a probable human carcinogen by the International Agency for Research on Cancer (IARC) and considered to be a proven human carcinogen by the German MAK Commission (Gaber et al., 2007). Most of the anilines are mild irritants to the eyes when in the liquid form but are highly toxic when absorbed through the skin, inhaled as vapour, or swallowed. Symptoms of over-exposure include confusion, weakness, disorientation, lethargy, convulsions, decrease in kidney enzyme activity, arrhythmias and shock. Death is uncommon but is usually due to cardiovascular collapse resulting in respiratory failure (TOXNET).

1.1.3 Environmental Factors

Not only do these compounds have detrimental effects on humans but short and long-term effects on the environment can be irreversible. All of the compounds studied are priority pollutants, being toxic to aquatic life in low concentrations. Salmonid fish,

considered to be the most environmentally sensitive species, have a LC_{50} for cresols of 6.2-8.4 mg/L (Kavitha and Palanivelu, 2005). Phenol at 1.0 mg/L or greater affects aquatic life (Veeresh et al., 2005). Many phenols are highly toxic, highly stable and rather resistant to natural biodegradation and they continue to persist in the environment as a threat to the ecosystem (Juang and Tsai, 2006). All the phenols are expected to have high mobility in soil based on K_{oc} (organic carbon coefficient) values which are subject to change depending on conditions of study especially pH, iron content, and presence of organic matter (Kopinke et al., 1995; Shin et al., 1994; Briggs 1981; Boyd et al., 1983; Scott et al., 1983; Swann et al., 1983). They are not expected to adsorb onto suspended solids and sediments in water. Volatization of phenol and m-cresol from water is not expected to occur while volatization of o-,p-cresol is expected to be slow from the water surface based on the respective Henry's Law constants and vapour pressures (Gaffney et al., 1987; Altschuh et al., 1999) (**Table 1.1**). Since such low concentrations of phenols have such detrimental effects on the ecosystem, stringent effluent guidelines are imposed to ensure the damage is minimized. Phenol is produced at about 6 million ton/yr worldwide with an ever increasing demand (Busca et al., 2008). The concentration of phenols in effluents typically ranges from 10.0 mg/L to 17.0 x 10^3 mg/L (Veeresh et al., 2005). COD contributions in these types of effluents for phenols can range from 40-80%, with petroleum refinery waste below 40% and coal conversion and coke oven effluents averaging 60% phenol, 30% cresols as mixed isomers (Cooper and Wheatstone, 1973; Fedorak and Hrudey, 1986; Nakhla and Suidan, 1995). Phenolic effluents from the various industries have been broken down as follows: refineries 6-500 mg/L, coking operations 28-3900 mg/L, coal processing 9-6800 mg/L, manufacturing of

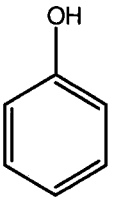
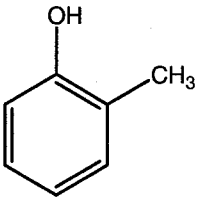
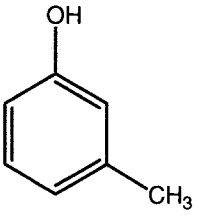
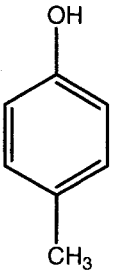
petrochemicals 2.8-1220 mg/L and other wastewater of pharmaceutical, plastic, wood products, paint, pulp and paper industries 0.1 – 1600 mg/L (Busca et al., 2008). Most discharge limits require a reduction to 0.5 mg/L (Tay et al., 2001). The Ministry of Environment and Forest (MOEF), in India imposed a maximum p-cresol discharge to surface water at 1.0 mg/L, while the World Health Organization (WHO) restricts p-cresol concentration to 0.001 mg/ L in potable water (Singh et al., 2008; WHO 1963). The European Union recommends that the limit for potable and mineral waters in effluents as 0.5μg/L and 0.5 mg/L, respectively, and surface waters for sewage systems as 1.0 mg/L (Busca et al., 2008; law no. 152/2006) A list of phenol discharges can be found in **Table 1.3**.

The aniline compounds have moderate to high mobility in soil (Gawlik et al., 1998; Swann et al., 1983; Briggs et al., 1981). These compounds are not expected to adsorb to suspended solids and sediment in the neutral form, but when protonated, which can occur under environmental pH's, binding to soil will occur. Based on their Henry's Law constants and vapour pressures, the anilines are expected to volatilize from moist soil but not dry (Jayasinghe et al., 1992) (**Table 1.2**). It has been reported that 80,000 and 475,000 ton/yr of aniline are produced in the US and China, respectively (Qi et al., 2002; O'Neil et al., 2000). It has also been reported that due to geological properties in north-eastern China the crude petroleum oils contain above-average nitrogen containing compounds such as aniline and pentylamine (Wang et al., 2007; Wang and Zheng, 1999). The government of China has put a maximum discharge limit of 1.0 mg/L of aniline in effluents (GB 8978-1996, National Standard of China) (Xing-yu et al., 2007). Aniline releases in North America can be found in **Table 1.3,** for reporting facilities.

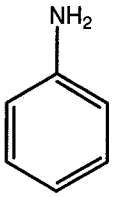
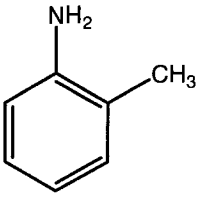
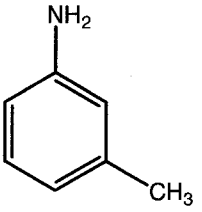
1.2 Current Wastewater Treatment Methods and Methods Being Investigated

The ever increasing concern for the environment and the implementation of more stringent effluent guidelines has prompted the scientific community to investigate new, innovative, cost-effective methods for the removal of phenols and anilines. Many of the methods in use today have changed little in the past decades and can suffer from high cost, low efficiency, harsh conditions, are energy intensive, are high in maintenance, the formation of hazardous by-products, or applicability to only low level concentrations of pollutant (Klibanov et al., 1980). Current treatment methods used are divided into three categories chemical, physical, and biological treatment methods.

Physical and chemical methods used include activated carbon, ozone oxidation, Fenton oxidation, photocatalytic oxidation, electrochemical oxidation, wet air oxidation and incineration (Gu et al., 2008; Suarez-Ojeda et al., 2008; Singh et al., 2008; Kavitha and Palanivelu, 2005; Beltran et. al., 1990). However these methods can be very energy intensive, costly, unselective in terms of pollutant removed, high maintenance and generate toxic by-products (Klibanov et al., 1980). As pollutant concentrations increase the conditions in the reactor also become increasingly hazardous due to the increase in temperature, pressure, and reactive oxidants. Incineration is appropriate for effluents with TOC > 100g/L but is very energy intensive and not very "green" in that the dust dissemination into the atmosphere and dioxin production is hazardous to the environment (Sudrez-Ojeda et al., 2008). Activated carbon and other adsorption techniques are costly and pollutants have only been removed not converted, also factors such as regeneration

Table 1.1: Chemical and Physical Properties of Phenols				
Chemical	**Phenol**	**o-Cresol**	**m-Cresol**	**p-Cresol**
Molecular Structure				
Synonyms	hydroxyl-benzene	2-methylphenol 2-hydroxytoluene o-cresylic acid	3-methylphenol 3-hydroxytoluene m-cresylic acid	4-methylphenol 4-hydroxytoluene p-cresylic acid
CAS Registry	106-95-2	95-48-7	108-39-4	106-44-5
Molecular Formula	C_6H_6O	C_7H_8O	C_7H_8O	C_7H_8O
Molecular Weight	94.11 [(1)]	108.14 [(1)]	108.14 [(1)]	108.14 [(1)]
Colour	Colourless acicular crystals [(1)]	White crystals, becoming dark with age[(1)]	Colourless, yellowish liquid [(1)]	Crystalline solid [(1)]
Odour	Distinct aromatic [(1)]	Phenolic odour [(1)]	Phenolic odour [(1)]	Phenolic odour [(1)]
Boiling Point °C	181.75 [(1)]	191.0-192.0 [(1)]	202.0 [(1)]	201.8 [(1)]
Melting Point °C	40.91 [(1)]	30.0 [(1)]	11.0-12.0 [(1)]	35.5 [(1)]
Density	1.071 @ 20 °C [(2)]	1.047 @ 20 °C [(2)]	1.034 @ 20 °C [(2)]	1.0185 @ 40 °C [(2)]
pK_a	9.99 [(2)]	10.29 [(3)]	10.09 [(3)]	10.26 [(4)]
Log Kow	1.46 [(5)]	1.95 [(5)]	1.96 [(5)]	1.94 [(5)]
Vapour Density (Air = 1)	3.24	3.72	3.72	3.72
Vapour Pressure mm Hg @ 25 °C	0.35	0.299	0.11	0.11
Henry's Law Constant Atm-cu m/mol @ 25 °C	3.33×10^{-7} [(6)]	1.2×10^{-6} [(6)]	8.6×10^{-7} [(7)]	1.0×10^{-6} [(6)]
Water Solubility	1g/ 15 mL of H_2O [(8)]	40 parts H_2O [(8)]	40 parts H_2O [(8)]	2.5g in 100 mL @ 50°C [(8)]

[(1)] O'Neil et al., 2001 [(2)] Lide et al., 2002, [(3)] Shiu et al., 1994 [(4)] Pearce and Simkins 1968 [(5)] Hansch et al., 1995 [(6)] Gaffney et al., 1987 [(7)] Altschuh et al., 1999 [(8)] Windholz et al., 1983.

Table 1.2: Chemical and Physical Properties of Anilines				
Chemical	**Aniline**	**o-Toluidine**	**m-Toluidine**	**p-Toluidine**
Molecular Structure				
Synonyms	aminobenzene	2-aminotoluene	3-aminotoluene	4-aminotoluene
CAS Registry	62-53-3	95-53-4	108-44-1	106-49-0
Molecular Formula	C_6H_7N	C_7H_9N	C_7H_9N	C_7H_9N
Molecular Weight	93.13 [(1)]	107.16 [(1)]	107.16 [(1)]	107.16 [(1)]
Colour	Colourless when pure to brown upon exposure	Light yellow becoming brown on exposure to light	Colourless to light yellow	Lustrous plates or leaflets
Odour	Aromatic	Aniline like	Aniline like	Aromatic
Boiling Point °C	184.1 [(2)]	200.3 [(2)]	203.3 [(2)]	200.4 [(2)]
Melting Point °C	-6.0 [(2)]	-16.3 [(2)]	-31.2 [(2)]	44-45 [(2)]
Density @ 20°C	1.0217 [(2)]	1.008 [(2)]	0.9889 [(2)]	1.046 [(2)]
pK_a @ 25 °C	4.63 [(3)]	4.44 [(3)]	4.73 [(3)]	5.08 [(3)]
Log Kow	0.90 [(4)]	1.32 [(4)]	1.40 [(4)]	1.39 [(4)]
Vapour Density (Air = 1)	3.3	3.7	3.9	3.9
Vapour Pressure mm Hg @ 25 °C	0.49	0.26	0.3	0.286
Henry's Law Constant Atm-cu m/mol @ 25 °C	2.02×10^{-6} (5)	2.2×10^{-6} estimated	2.8×10^{-6} estimated	2.02×10^{-6} (5)
Water Solubility	1g / 28.6 mL of H_2O [(6)]	Slightly soluble (6)	Slightly soluble (6)	135 parts water (6)

[(1)] O'Neil et al., 2001 [(2)] Lide et al., 2002 [(3)] Weast et al., 1977 [(4)] Hansch et al., 1995 [(5)] Jayasinghe et al., 1992 [(6)] Windholz et al., 1983.

Table 1.3: Chemical Discharges for Reporting Facilities in 2006						
	TRI Database 2006			**NPRI Database 2006**		
Chemical	**Total On-site Disposal or Other Release (kg)**	**Total Off-site Disposal or Other Release (kg)**	**Total On- and Off-site Disposal or Other Release (kg)**	**On – Site Release (kg)**	**Disposal On and Off-site (kg)**	**Recycled (kg)**
Phenol	2,651,149	1,223,277	3,206,018	722,000	137,000	348,000
o-Cresol	5,565	1,177	6,741	N.R.	N.R.	N.R.
m-Cresol	21,738	32,484	54,222	N.R.	N.R.	N.R.
p-Cresol	17,211	15,246	32,457	N.R.	N.R.	N.R.
Cresol (mixed isomers)	461,190	14,911	476,101	39,000	751	40,000
Aniline	400,305	244	400,549	0	21,000	0
o-Toluidine	8,526	23	8,549	N.R.	N.R.	N.R.
m-Toludine	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.
p-Toluidine	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.

USA EPA TRI (Environmental Protection Agency Toxic Release Inventory) NPRI (National Pollutant Release Inventory – Canada) N.R. – No Report Generated.

and disposal have to be considered. Adsorption capacity can vary based on the nature of pollutant, pore size, and solution conditions (Busca et al., 2008; Laszlo, 2005). Other physical/chemical methods are designed to mineralize the organics to CO_2 and H_2O. Wet air oxidation (WAO) and catalytic wet air oxidation (CWAO) are based on O_2 oxidizing properties. WAO involves the mass transfer of oxygen from the gas to the liquid phase providing the rate limiting step for the overall process. This process is influenced considerably by its reaction parameters: temperature, partial pressure, pH of solution. From the numerous studies done it has been observed that complete mineralization of phenol may not be possible due to the generation of low MW oxygenated compounds (acetic acid, propionic acid, methanol, ethanol, acetaldehyde) which are resistant to oxidation (Busca et al., 2008; Santos et al., 2006). CWAO generally utilizes a

heterogeneous catalyst system to abate the costly, high pressure, energy intensive conditions of WAO, the catalyst usually involving a transition metal such as Cu, Fe, Mn, Co, or noble metals Pt and Ru. Heterogeneous catalysts are generally easier to remove after treatment. Pt_xAg_{1-x}-MnO_2/CeO_2 is one such catalyst that has achieved promising results for phenol degradation (Hamoudi et al., 2000). Oxidation by ozone is achieved by O_3 attacking the nucleophilic and unsaturated bonds of organic compounds. O_3 is ideal because of its high reduction potentials at both acidic and basic pH's. Ozonolysis can achieve 100% mineralization, usually with the aid of a heterogeneous catalyst, but it remains a costly method and is not applied to large-scale operations (Beltran et al., 1990; Busca et al., 2008). Fenton oxidation, by reacting H_2O_2 with Fe^{2+} in acidic pH (Fe^{3+} in acidic pH with H_2O_2 to produce $HO_2\bullet$ radicals to regenerate the catalyst) generates $^{\bullet}OH$ radicals which are highly reactive and react almost indiscriminately to mineralize organic compounds. H_2O_2 is cheap, safely stored and the reaction can be run in non-pressurized reactors at or near room temperature. The major disadvantage is that efficient conversion is based upon a stoichiometric excess of H_2O_2 which will require the removal and disposal of large quantities of ferric salts if the organic content is high (Kavitha and Palanivelu, 2005; Busca et al., 2008). Other chemical oxidations with chlorine, chlorine dioxide, or potassium permanganate are not environmentally friendly due to the generation of chlorinated organic compounds or Mn dispersion. In addition to the reagents' being costly, the pH must be tightly regulated (Busca et al., 2008). More environmentally friendly methods have been tested such as the ferrate (IV) ion. However its redox potential decreases with increasing pH and with phenol only a maximum of 80% degradation was observed with a 5:1 ratio of ferrate to phenol at pH 9.2. Increasing

the pH to 11.0 greatly decreases phenol conversion due to the decrease in reduction potential and perhaps the dissociation of phenol to phenolate ion (Graham et al., 2004). In a separate study ferrate (IV) was shown to oxidize phenols and phenolic endocrine disrupters (Lee et al., 2005). Electrochemical oxidation can occur through indirect electro-oxidation or direct anodic oxidation. Indirect electro-oxidation can be done using chlorine and hypochlorite to destroy pollutants but usually requires high salt concentrations, generates chlorinated intermediates and, if salt concentrations are low, large amounts of chlorine must be added. An electro- Fenton method can be applied in the 2-electron reduction of O_2 to H_2O_2 and it can be accomplished at the right cathodic potential of certain electrodes. The H_2O_2 can then be used as mentioned in Fenton oxidation to generate •OH radicals by the addition of Fe^{2+} , the H_2O_2 generated may be more cost-effective and efficient then chemical dosing (Chen, 2004; Panizza and Cerisola, 2001; Busca et al., 2008). Through the same concept, electrically generated O_3 can be produced. Metals have also been employed as mediators in which they are oxidized on the anode and then attack organic pollutants directly or generate reactive –•OH radicals that can attack the pollutant (Farmer et al., 1992). Direct anodic oxidation occurs directly on the anode through the generation of physically chemisorbed "active oxygen" (•OH radicals, or O_2 in oxide lattice MO_{x+1}). Usually a high over-potential is required for total oxidation since generation of O_2 can also occur on the anode (Busca et al., 2008). Boron-doped diamond (BDD) electrodes and Ti/SnO_2-Sb anodes have also shown to be able to completely mineralize phenols, usually in the potential region of oxygen evolution (•OH radical generation) but also in the potential region before oxygen evolution (stability of water) (Panizza and Cerisola, 2005; Li et al., 2005). Photocatalytic

oxidation has also been investigated in the destruction of wastewater constituents, usually in the presence of the photocatalyst TiO_2-anatase (Thompson and Yates Jr., 2006). While the photocatalyst is inexpensive it has a moderately large band gap (3.0-3.2 eV) utilizing only a small fraction (λ < 380 nm, UV region) of the solar spectrum that is absorbed. Lastly, supercritical water gasification which converts phenols to H_2, CO, CO_2 and CH_4 requires pressurization and high energy input (Busca et al., 2008).

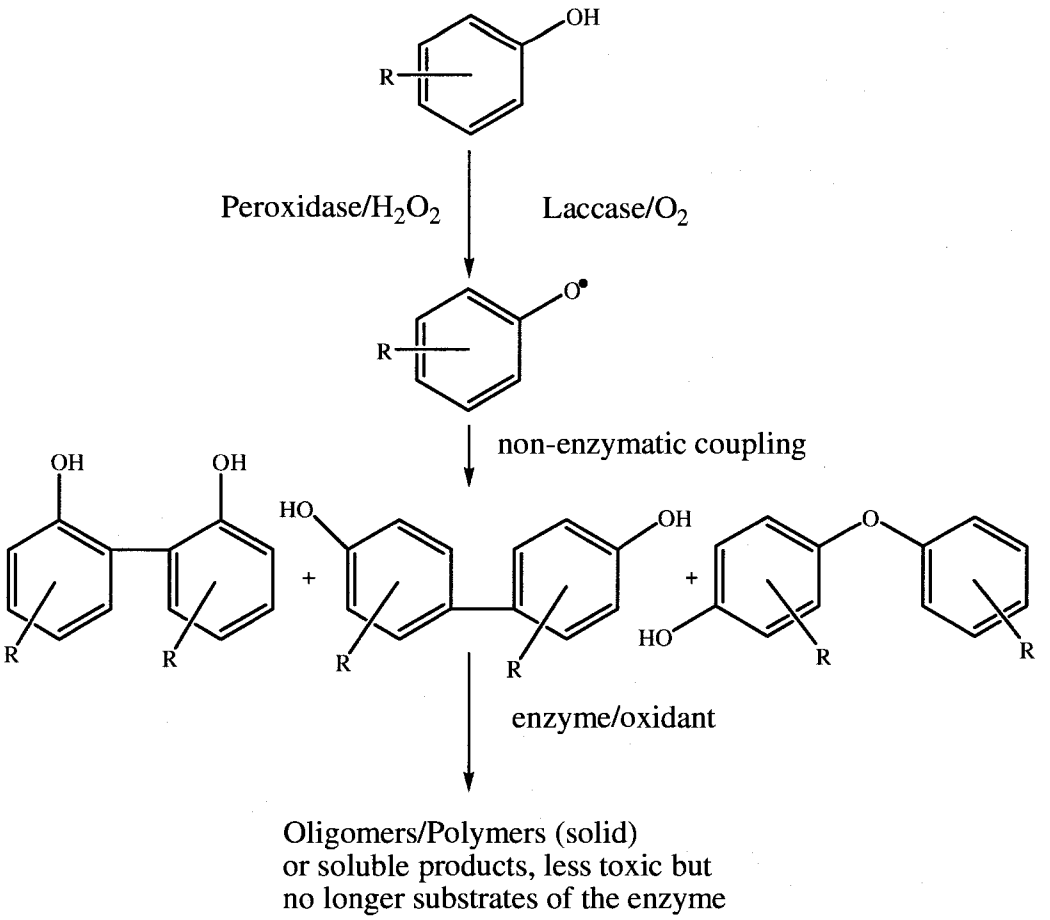
Current biological treatment methods include: activated sludge, bio-oxidation lagoons, aerated lagoons, oxidation ditch and rotating biological contactors. For large-scale operations biological treatment as an activated sludge process has been incorporated more often when trying to achieve complete elimination (Ryan et al., 2005; Bai et al., 2007). However this is often dependent on the reactor type and design. It is known that microbes are sensitive to both anilines and phenols which inhibit the growth rate of species that have the metabolic capabilities of digesting them. Therefore both phenol and aniline must be kept below the toxic limit and the microbes must be acclimatized to the effluent environment which can be a lengthy process. For both the anilines and phenols numerous microbial strains have been cited to work, however many studies focus only on one microbial species and it is possible that co-aggregative cells can lead to metabolitic advantages over single cells (Busca et al., 2008). When multiple strains are present often the focus is only on the removal of a single compound or single group of compounds and not complex mixtures representative of real wastewater effluents (Xing-yu et al., 2007). Microbial strains capable of degrading phenol include: *Pseudomonas putida*, *Pseudomonas fluorescens*, *Acinetobacter*, *Trichosporon cutaneum*, *Candida tropiccalis*, and even some fungal strains such as *Nocardia hydocarbonoxydans*. Studies with aniline

have included *Pseudomonas* strains isolated from activated sludge from a Chinese petrochemical wastewater treatment facility due to the higher NAC's in the region as well as studies with mixed strains of various anaerobic and aerobic sources (Gu et al., 2008; Wang et al., 2007; Carcalho et al., 2008). It was also found in sequencing batch reactors that after a 190 d acclimatization period, required for NAC's (Nitrogen-containing Aromatic Compounds), bacterial strains of *Bateriodetes*, *β-Proteobacteria* and Candidate division TM7 were greatly increased from day 1 and dominated the consortium in the reactors and contributed to the degradation of NACs (Xing-yu et al., 2007). The design and type of reactor used will ultimately be dependent on the industry, effluent type, space, and volume required to be treated in a turnover period. All types of biological treatment are dependent on keeping the micro-organisms alive; therefore incubation temperature, salinity, pH, and aeration are all parameters that must be maintained. Today a number of combined and sequential processes are also being investigated involving the incorporation of either physical or chemical methods with biological treatment or two different physical/chemical methods.

1.3 Enzymatic Treatment

Enzymatic treatment was first introduced in the early 1980's by Klibanov and co-workers in their work with peroxidases and Bollag and co-workers in their work with laccases (Klibanov et al., 1980; Bollag et al., 1979). Enzymatic treatment, regardless of oxidoreductase used (peroxidase, laccase or tyrosinase) all follow a central dogma in the removal of phenols and amines. Peroxidases and laccases (**Scheme 1.1**) catalyze the oxidation of phenols and aromatic amines into radical species in the presence of oxidant (H_2O_2 for peroxidases and O_2 for laccases). These highly reactive radical species then

couple non-enzymatically to produce dimers. Dimer products, still aromatic containing -OH or $-NH_2$ functionality, are often better substrates of the enzyme, therefore, they can be further oxidized into higher oligomers (Yu J. et al., 1994). This cycle should continue until the products become hydrophobic enough that they precipitate out of solution and can be removed by filtration and sedimentation methods. When comparing conventional biological treatment to enzymatic treatment the following potential advantages of the latter include: operation over a broader range of pH, salinity, on a more diverse range of compounds including some toxic to microbes (biorefractory), short contact time required, have no shock loading effects associated with start up and shut down, reduction of biomass for further disposal, and easier to maintain (simpler control) (Taylor et al., 1996). With respect to chemical and physical means, enzymatic treatment has the potential to operate under milder or less corrosive conditions (usually increase with increasing pollutant concentration), consumption of less oxidant due to catalytic nature, reduction in solid waste compared to activated carbon or adsorptive materials, works on trace level organics not normally removed (Taylor et al., 1996; Nicell et al., 1993). The major disadvantage to the enzyme-based treatment method is the cost of the enzymes, which are becoming increasingly more affordable due to methods; to reduce inactivation and increase efficiency combined with the development of new technology for large-scale enzyme production, purification and stabilization. Tyrosinases operate through an alternative method described later.



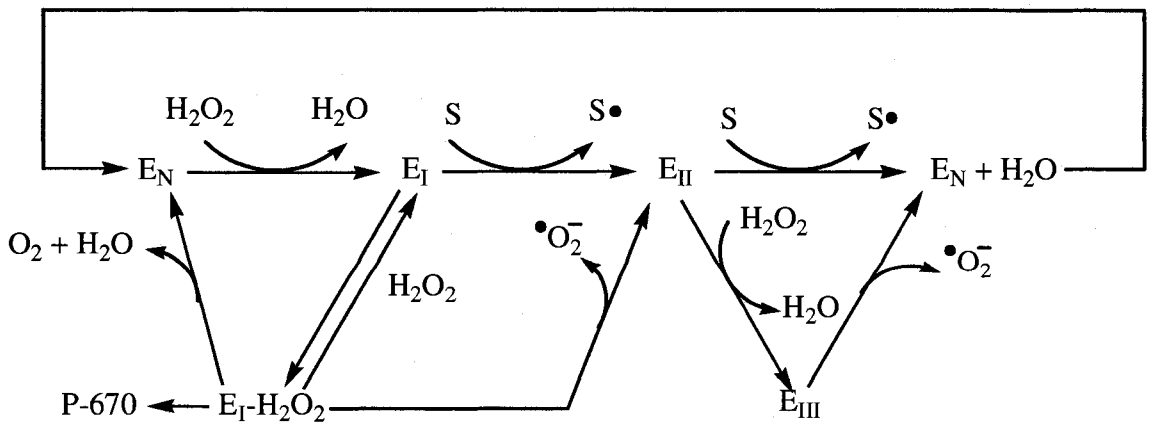
Scheme 1.1: Enzymatic Treatment for Phenols (and Anilines) Reproduced from Taylor et al., (1998).

1.4 Peroxidases

Peroxidases were one of the first enzymes discovered in the 19th century and have been found in plants, animals, bacteria and fungi (Dunford, 1999). The plant peroxidases are divided into three classes. Among the class III, or secretory plant peroxidases, horseradish peroxidase (HRP) has been most extensively studied for its possible application in enzymatic treatment. Soybean peroxidase, another class III peroxidase, has been receiving attention lately because of its high catalytic efficiency even in its crude form, obviating the cost of purifying the protein. The seed coat where the majority of the

enzyme resides can simply be washed with buffered water to extract the crude enzyme (Bassi et al., 2004). Many fungal or class II peroxidises such as *Arthromyces ramosus* peroxidase (ARP) have been investigated because of similar specific activity and substrate profile to HRP while being secreted extracellularly in large quantities to the liquid medium (Patapas et al., 2007; Villalobos & Buchanan, 2002). Other possible plant peroxidases include bitter gourd, tomato hair root (*Momordica charantia*) and turnip (*Brassica napus*) (Akhtar and Husain, 2006; Gonzalez et al., 2008; Quintanilla-Guerrero et al., 2008). Other fungal sources investigated include *Coprinus macrorhizus* (CMP) also known as *Coprinus cinereus* (CiP) and *Coprinus lagopus* (Al-Kassim et al., 1994; Ikehata et al., 2005; Ikehata et al., 2002).

Peroxidase catalyzes the dehydrogenation of phenols and anilines in the presence of H_2O_2 following a modified ping-pong or a peroxidase ping-pong mechanism as shown in **Scheme 1.2.** (Dunford, 1999). The native form of the enzyme (E_N) is oxidized in a 2-electron step while reducing H_2O_2 to H_2O forming Compound I (E_I) (oxy-ferryl state) (**Equation 1**). E_I is returned to the native form following two successive one-electron steps passing through an intermediate, Compound II (E_{II}) (**Equations 2 & 3**).



Scheme 1.2: Catalytic Cycle for peroxidases. EN – native enzyme E_I - compound I, E_{II} – compound 2, S - phenol or aniline species (Dunford, 1999; Wright et al., 1999).

Both E_I and E_{II} accept an electron from phenols or anilines, converting them to free radicals (S•) (uncharged, highly reactive intermediate due to the unpaired electron) (Wu et al., 1999; Dunford, 1999). Although peroxidases seem efficient in removing pollutants from effluents, a certain amount of control (approximate concentration of pollutants known) is required since peroxidases are subjected to three known suicide pathways. When hydrogen peroxide is in excess or pollutant concentrations are low, the E_{II} form of the enzyme is converted to the E_{III} form (Compound III), which is not catalytically relevant due to it slow decay rate back to E_N (**Equations 4 & 5**) (Dunford,1999; Wright and Nicell, 1999). The E_{III} can be reduced back to the E_I form by a one-electron reduction generating a radical. The E_I form can now either be reduced back to the native form through a series of reactions or form the terminally inactive verdohemoprotein, referred to as P-670, due to a shift in absorbance from 405 nm to 670 nm (**Equations 6-8**) (Dunford ,1999; Wright and Nicell, 1999; Villalobos and Buchanan, 2002).

$$E_N + H_2O_2 \xrightarrow{k_1} E_I + H_2O \quad (1)$$

$$E_I + S \xrightarrow{k_2} E_{II} + S\bullet \quad (2)$$

$$E_{II} + S \xrightarrow{k_3} E_N + S\bullet + H_2O \quad (3)$$

$$E_{II} + H_2O_2 \longrightarrow E_{III} + H_2O \quad (4)$$

$$E_{III} \longrightarrow E_N + O_2^{\bullet-} + H^+ \quad (5)$$

$$E_{III} + S \longrightarrow E_I + S\bullet \quad (6)$$

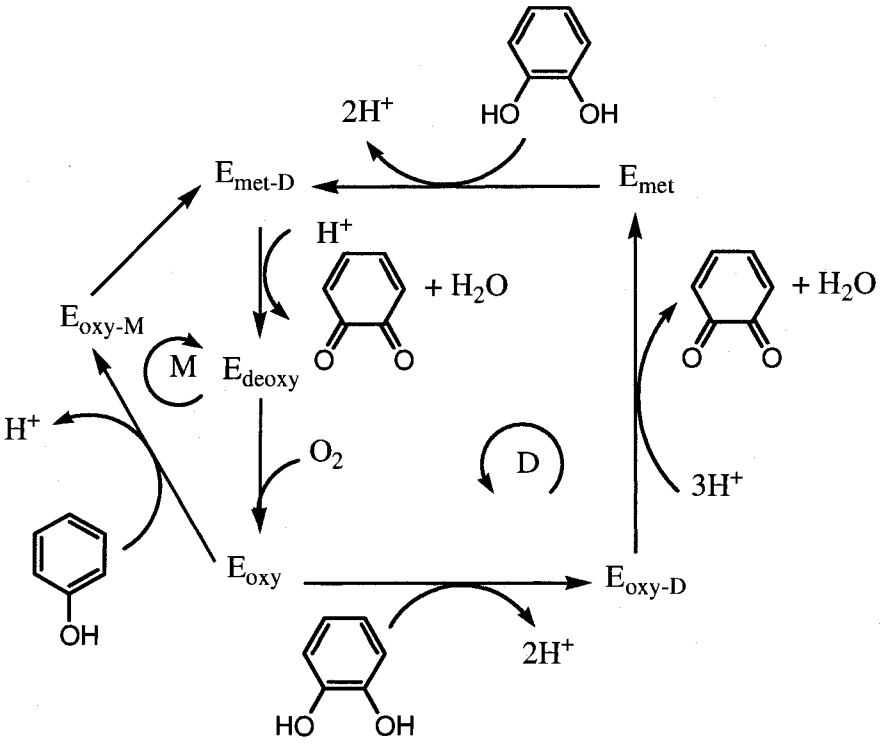
$$E_I + H_2O_2 \longrightarrow E_N + O_2 + H_2O \quad (7)$$

$$E_I + H_2O_2 \longrightarrow \text{P-670} \quad (8)$$

All peroxidases are susceptible to H_2O_2 inactivation, some more than others. In order to avoid such inactivation, stepwise addition of hydrogen peroxide has been suggested when high concentrations are required (Ibrahim et al., 2001). Other types of inactivation will be discussed in Chapter **4.**

1.5 Tyrosinases

Tyrosinase [monophenol, dihydroxyphenylalanine (DOPA): dioxygen oxidoreductase EC. 1.14.18.1] is a multicopper oxygenase containing binuclear coupled Cu's in the active site. This enzyme catalyzes the ortho-hydroxylation of monophenols as well as the two-electron oxidation of o-diphenols to o-quinones (Solomon et al., 1996). Tyrosinase is widely distributed in microorganisms, animals and plants. Recently, mushroom tyrosinase has received a lot of attention because of its availability. The catalytic cycle can be observed in **Scheme 1.3** in which the oxidation of monophenols is shown as the enzyme passes through 4 intermediates (E_{deoxy}, E_{oxy}, $E_{oxy\text{-}D}$, $E_{met\text{-}oxy}$) and the o-diphenols are converted to highly reactive o-quinones while passing through five intermediate states (E_{deoxy}, E_{oxy}, $E_{oxy\text{-}D}$, E_{met}, $E_{met\text{-}D}$). The following states are denoted by the states of the coupled binuclear copper sites as follows: deoxy [Cu(I) Cu(I)], mixed-valent half met [Cu(II) Cu(I)], EPR non-detectable met [Cu(II)-Cu(II)], EPR dimer [Cu(II)—Cu(II)] and oxy [Cu(II) O_2^{2-} Cu(II)] (Solomon et al., 1996). In nature, these o-quinones then undergo non-enzymatic polymerization to form the pigment melanin.



Scheme 1.3: Catalytic Cycle for Tyrosinases. The hydroxylation of monophenols (M) 2. dehydrogenation of o-diphenols (D) to o-quinones; M-monophenol D-diphenol (Solomon et al., 1996; Decker et al., 2007).

This concept is then applied to enzymatic treatment scheme with the o-quinones reacting non-enzymatically compared to the non-enzymatic radical coupling involved in polymerization with peroxidases and laccases. Mushroom tyrosinase was proposed in the "dephenolization" of industrial wastewater by Atlow and co-workers in 1984 (Atlow et al., 1984). Others have characterized and successfully transformed a number of phenols using tyrosinase (Ikehata and Nicell, 2000; Wada et al., 1995). It has also been reported that the products generated by phenol compounds have a tendency to react with the amino-group containing polymers such as chitosan and polyethylenimine (PEI) (Wada et al., 1995). Other studies have investigated the effectiveness of immobilized tyrosinase and as well as the combined action of adsorbtion by chitosan beads (Wada et al., 1993; Yamada et al., 2005). Tyrosinases have been reported to generate phenol products that

are primarly soluble, unless chitosan was added to promote coagulation or flocculation (Ikehata and Nicell, 2000). Even 10 mM phenol was reported not to produce visible precipitation. In such case methods to remove the transformed products will have to be developed as well as testing the toxicity of these products before it is considered as a viable alternative to peroxidases and laccases. Like laccase, tyrosinase uses O_2 to activate the enzyme, unlike peroxidases that require the addition of H_2O_2.

1.6 Laccase

1.6.1 Laccases

Laccases (E.C. 1.10.3.2, *para*-benxenediol:oxygen oxidoreductases, Lc) are multicopper (blue copper) oxidases that can be defined by their spectroscopy, reactivity, homology and are widely distributed in nature (Solomon et al., 1996; Tadesse et al., 2008). Other well characterized multicopper oxidases include ascorbate oxidase (AO) and ceruloplasmin (Cp) (Solomon et al., 1996). More recently characterized but less defined are phenoxazinone synthase (PHS), bilirubin oxidase (BO), dihydrogen oxidase (DHGO), sulochrin oxidase (SO) and Fet3 (Solomon et al., 1996). These multicopper oxidases contain at least 4 Cu atoms and catalyze 4 one-electron oxidations of substrates, typically phenols, anilines, aminophenols, methoxy-substituted phenols, polyphenols, polyamines, lignins as well as some inorganic ions ($[Mo(CN)_8]^{-4}$, $[Fe(CN)_6]^{-4}$, $[Os(CN)_6]^{-4}$, $[W(CN)_8]^{-4}$) while reducing O_2 to H_2O and by-passing a stage of H_2O_2 production (Lee et al., 2002; Morozova et al., 2007; Shleev et al., 2006). Laccase is the simplest of these multicopper oxidases containing only 4 Cu atoms in its active site that are distinguished/characterized based on their spectroscopic and/or magnetic properties in their oxidised form (Solomon et al 1996; Palmer et al., 1999; Matera et al., 2008). Due to its broad substrate specifity

as well as its ability to oxidise substrates with redox potentials above its own, it has been widely investigated in recent years for applications in oxygen cathodes in biofuel cells (Barton et al., 2001; Barton et al., 2004), biosensors (Shleev et al., 2006; Ghindilis et al., 1992; Gomes et al., 2004), biodegradation/ pulp bleaching (Geng et al., 2004), green organic syntheses including conductive polymers (Karamyshev et al., 2003; Niedermeyer and Lalk, 2007; Witayakran et al., 2007) and bioremediation/wastewater treatment (Steevensz et al., 2008; Modaressi et al., 2005; Ghosh et al. 2008; Kim & Nicell, 2006). Laccases are glycoproteins with MW 50 -130 kDa, first described by Yoshida from the lacquer tree *Rhus vernicifera*, by far the most widely studied plant laccase. Since then laccases have been reported in higher plants, almost all fungi tested for them and more recently in insects and prokaryotes (Riva 2006, Yoshida, 1883). Plant laccases are monomeric with 22-45% glycosylation and reduction potentials from ~300-500 mV (T1), usually accounting for their lower reactivity in comparison to the fungal laccases that have higher reduction potentials between 400 -790 mV and generally lower carbohydrate (mannose. N-acytlglucosamine, galactose) content of 10-20 % (Morozova et al., 2007; Solomon et al., 1996). The carbohydrate moiety is thought to aid in maintaining the stability of the protein globule and at least one case has been documented where deglycosylation obviated enzyme activity (Ko et al., 2001). Excluding *Rhus vernicifera*, data on higher plant laccases (*Rhus succedanea*, *Acer pseudoplatanus*, *Pinus taeda*, *Populus euramericana*, *Liriodenfron tulipifera*, *Nicotiana tobacco*, *Lolium perenne*, *Zeas mays* – partially characterized) are scarce and only partially characterized while the majority of laccases studied have been isolated from white rot fungi (mainly *Trametes* (*Coriolus*) *versicolor*, *T. hirsute* (*C. hirsutus*), *T. ochracea*, *T. villosa*, *T. gallica*, *Cerrena*

maxima, *Coriolopsis polyzona*, *Lentinus tigrinus*, *Pleurotus eryngii*). Most white rot fungi have both intracellular and extracellular laccases but ~ 90% of the enzyme is extracellular, assumed to be associated with their physiological functions. Intracellular laccases are thought to help protect cells in the transformation of low MW phenols while the extracellular laccases are involved in pigment formation, lignin degradation, and detoxification (Solomon et al., 1996). A putative role in lignin degradation has not been established since *in vivo* peroxidases also play a role. Fungi without laccase can still degrade lignin and *in vitro* laccase studies have shown it has the ability to degrade high MW lignin and repolymerize low MW compounds. Dimer models have been shown to be oligomerized or cleaved depending on the compound of interest (Solomon et al., 1996). It has been proposed that laccase may be responsible for removing potentially toxic phenols during lignin degradation working synergistically with multiple enzymes, *in vivo* in the lignin degradation process. Most fungi produce many isoforms of the enzyme, some of which are only detectable under certain culturing conditions or in the presence of specific inducers in the medium (Galhaup & Haltrich, 2001; De Souza et al., 2004; Dong et al., 2005; Palmieri et al., 2003). The function of multiple isoforms is not fully understood at this point. Like plant laccases most fungal laccases are monomeric, but enzymes consisting of several subunits have been documented. *T. villosa* is often isolated as a homodimer (Yaver et al., 1996). *Monocillium indicum* is composed of three subunits of 24, 56, and 72 kDa making it a heterotrimer (Thakker et al., 1992). Homotetramers and homotrimers of the enzyme or laccase-like enzymes have also been observed. Laccase molecules are usually monomeric consisting of three cupredoxin-like domains connected one after another twisted into a tight globule. Dimensions of *C. cinereus* laccase are 70 x

50 x 45 Å and for *T. versicolor* are 65 x 55 x 45 Å, determined through their crystal structures (Ducros et al., 1998; Piontek et al., 2002). The tri-cluster (T2/T3) is found between the first and third domain, while the T1 Cu is found in the third domain, all ligated to amino acids (Solomon et al., 1996; Piontek et al., 2002). Amino acids from both the second and third domains form the substrate binding pocket. Disulfide bridges are responsible for connecting the domains (*T. versicolor* Cys8-Cys488 C-end to domain 1, Cys117-Cys205 Domain 1-2) (Piontek et al., 2002).

Some laccases with unique and potentially useful characteristics for enzymatic treatment have been identified such as: thermostable laccase from *Pycnoporus sanguineus* (SCC 108) optimum temperature of 55 °C and a half life of 170 min at 75°C (Litthauer et al., 2007); Evidence of halotolerant-alkaline laccase in *Streptomyces psammoticus,* with optimum pH of 8.5 and resistant to NaCl concentrations up to 1.2 M (Niladevi et al., 2008); finally studies being done to assess the feasibility of using laccase in water-miscible solvents (Rodakiewicz-Nowak et al., 2000). For treatment regimes it is unlikely that a single enzyme will be suitable for all situations.

1.6.2 Structural Features

Despite the fact that laccase is the simplest of the multicopper oxidases, it is still a very complex system. Spectroscopic studies, sequence alignments and crystal structure comparisons have shown that all multicopper oxidases contain a minimum of one type 1 (T1), one type 2 (T2) and two type 3 (T3) Cu's. These Cu sites are defined by spectroscopic and/or magnetic properties that they exhibit in the oxidized (Cu^{2+}) state (Solomon et al., 1996; Palmer et al., 1999; Lee et al., 2002; Quintanar et al., 2005; Huang et al., 1999; Zoppellarno et al., 2001). The T1, "blue" Cu, has an intense absorption at

600-610 nm ($\epsilon \approx 5000\ M^{-1}\ cm^{-1}$) and small parallel hyperfine coupling in the paramagnetic resonance (EPR) ($<100 \times 10^{-4}\ cm^{-1}$). The T2, normal Cu, has no noticeable features in the visible or circular dichromism (CD) spectrum typical of a normal tetrahedral Cu with parallel coupling ($>160 \times 10^{-4}\ cm^{-1}$). The T3, coupled binuclear, Cu has two Cu^{2+} ions coupled antiferromagnetically by a bridging hydroxide, making them a diamagnetic spin coupled pair, with no EPR signal but still having a visible absorption at 330 nm ($\epsilon \approx 5000\ M^{-1}\ cm^{-1}$) (Matera et al., 2008; Solomon et al., 1996; Palmer et al., 1999). A plethora of multicopper oxidase crystal structures have been compared and have shown that the T2 and T3 Cu are in close proximity to each other forming a Cu tri cluster (~ 4-5 Å) as well as having similar Cu coordination (between the multicopper oxidases). However, significant differences in T1 ligation have been observed resulting in varying geometries which was previously thought to be involved in tuning the redox potential of the T1 Cu (Palmer et al., 1999). In a comparison of more than 100 laccases, four highly conserved regions were identified (Kumar et al., 2003). Contained within these regions are ten His, one Cys that form the ligation environment for the Cu ions in the active site (**Figure 1.1**). The T1 Cu is located 12-13 Å away from the tri-cluster and is the site where substrates are oxidized, while O_2 reduction to H_2O occurs at the tri-cluster (more later). The T1 redox potentials for many laccases are known ranging from 430 – 790 mV, but only two redox potentials for the T2 Cu site have been reported, *R. vernicerfera* (390 mV) (plant) and *T. hirsute* (400 mV) (fungal) (Reinhammer & Vanngard 1971; Shleev et al., 2005). T3 redox potentials for *R. vernicerfera* (460 mV) and *T. versicolor* (785 mV) have also been reported but the addition of F ions lowers this in *T. versicolor* by 210 mV due to its strong interaction with the T2/T3 tri-cluster

(Morozova et al., 2007; Reinhammer & Vanngard, 1971; Reinhammer, 1972). Laccases are often divided into high (730- 790 mV), medium (470- 730 mV) and low (< 470 mV) potential enzymes based on their T1 Cu redox potential. A number of sequence alignments show that fungal laccases with medium to high potential have a Leu or Phe in the axial position replacing the Met found in lower potential enzymes and in that of the classic T1 sites found in other multicopper oxidases; AO, human Cp, and plastocyanin (Pc). Both Leu and Phe have no functional group that can ligate to Cu as well as being too bulky for water to pass. Thus the Cu has a more trigonal planar geometry with two N_{His} and one S_{Cys} but no axial ligand (Palmer et al., 1999; Xu et al., 1999; Madzak et al., 2006). The nature of the T1 Cu site is of great interest due to both its unique electronic structure as well as its function as an electron-transfer center (Solomon et al., 1996). Both experimental and theoretical results show very little change in geometry when Cu^{2+} is reduced to Cu^{+} suggesting that this site has a low reorganization energy accompanying electron transfer (Palmer et al., 1999). It should be noted that the reduction potential of the T1 Cu site tends to be higher than that of aqueous Cu^{2+}. A summary of factors suspected to be involved in tuning the T1 Copper can be found in Table 1.4. The ligated Cu-S_{Cys} is highly covalent, and thus a possible pathway for electron transfer is through this residue and it appears as though its strength is inversely related to the axial Cu-S_{Met}, interaction in that the stronger the Cu-S_{Met} the weaker the Cu-S_{Cys} interaction (less covalent less efficient electron transfer) (Palmer et al., 1999; Xu et al., 1999). The Cu-S_{Cys} has been determined to be 30% more covalent by resonance Raman spectroscopy, thus indicating a stronger bond in fungal laccases with no axial ligand (Palmer et al., 1999). A series of studies were done to help elucidate the effect of this axial ligand and to

determine if the lack of it accounted for the higher redox potential and/or the significant differences in electronic structure compared to the classic Pc T1 Cu site. Through a series of mutations in *T. villosa* it was concluded that the axial ligand is important for catalytic properties but it did not account for the large gap in redox potential. The Phe corresponding to the T1 axial Met was replaced by both Leu (F463L) and (F463M) Met in *T.villosa* and assessed based on molecular and enzymological properties (Xu et al., 1999). F463L showed only minor alterations in EPR spectrum indicating little if any change in the T1 site suggesting that the replacement of the bulky π electron Phe with Leu had little impact on the T1 site (Xu et al., 1999). Similar results were observed when the reverse modification was made Leu to Phe in *Rhizoctonia solani* and *Myceliophthora thermophila* in that little difference in redox potential or electronic structure was observed. Consistent with this optimum pH, K_M and k_{cat} for syringaldazine and ABTS were unaltered (Xu et al., 1998; Xu et al., 1999). If alterations in the electronic spectrum of the T1Cu site were observed it could be anticipated that rate of substrate oxidation would be altered since this has been determined to be the rate-limiting step (ET at T1 site). The F363M mutant showed no perturbations in the T2 Cu site but both absorption and EPR results confirmed there were changes consistent with T1 Cu site becoming more like *Rhus vernicifera* and (Pc) plastocyanin. The reduction in E° supports these results with the reasoning that the additional Met ligand would stabilize the oxidized Cu^{2+} over the Cu^{+} site leading to a lower E° (Xu et al., 1999). Studies with other multicopper oxidases have demonstrated that replacing the Met axial ligand with Leu or Phe led to an increase in E° of 100 mV (Karlsson et al., 1989; Pascher et al., 1993). Even with this decrease in E° *T. villosa's* E° is still 680 mV which is considerably higher than AO and

most plant laccases. The inference through all this is that other factors must have to contribute to tuning the E° of multicopper oxidases such as: hydrogen bonding, solvent accessibility, and orientation of local dipoles (Xu et al., 1999). On top of the reduction in E° there was a 5-fold increase in K_M for syringaldazine and 38-fold increase for ABTS with a 2-fold increase in k_{cat} (Xu et al., 1999). The initial catalytic step involves the electron transfer from donor to acceptor in which the difference between E° of the two is the thermodynamic driving force for the redox reaction. The k_{cat} parameter is reflective of the rate-determining step, in this case the oxidation of substrate at the T1 Cu site (Xu et al., 1996). While k_{cat} may be representative of many steps, the fact that k_{cat} correlates between E° of the T1 in many series of substrates suggests that k_{ET} may be a major component to k_{cat} (discussed more in Results and Discussion). This is based on the fact that there is a decrease in E° in the F463M, decreasing the thermodynamic driving force of ET due to the altered ground state of the T1 Cu resulting in perturbations in the geometry at this site suspected to be changing the orbital overlap between the oxidizing substrate and half-occupied highest molecular orbital of the T1 Cu (Xu et al., 1999; Palmer et al., 1999; Xu 1996). Piontek and co-workers have proposed that the Cu-N bond length of one of the His ligated to the T1 Cu is responsible for E° tuning, in that increasing the bond length should decrease the contribution of free electrons of the nitrogen atom. When comparing *T. versicolor* (high potential) and *C. cinereus* (medium potential), the α-helix of *T. versicolor* is pulled more towards the first domain due to a H-bond between Glu460 and Ser113, lengthening the distance between Cu-N (Piontek et al., 2002). *C. cinereus* has a Met in the place of Glu460. When comparing high potential laccases with medium and low potential enzymes it appears as though the high potential

ones have Glu and Ser corresponding to the Glu460 and Ser113 in T. versicolor (Piontek et al., 2002; Bento et al., 2005).

Table 1.4 Summary of T1 Cu Tuning Factors		
	Plant	Fungal
T1 Redox Potential (mV)	< 500	400-800
T1 Axial Ligand	Met	Leu, Phe
Glycosylation (%)	22-45	10 - 20
Cu-N(His)	Short	Long

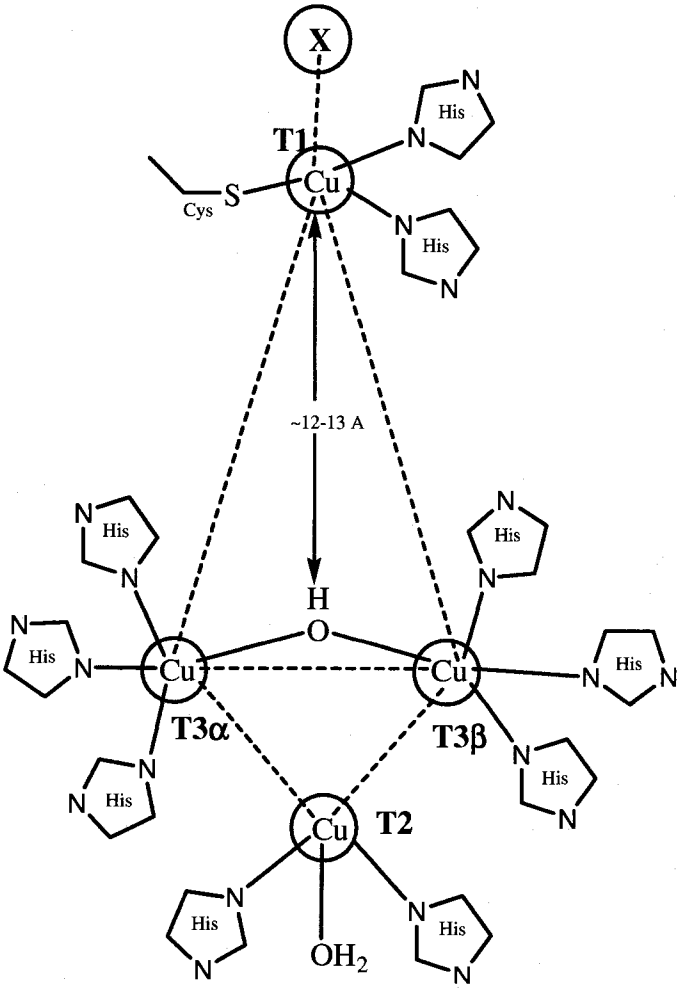


Figure 1.1: Active Site(s) of Laccase (not to scale) showing the 10 His and 1 Cys. **X** in the axial position of T1 is usually Met with ligation to T1 in plant sources usually, and Leu or Phe in fungal sources not ligated to T1. This difference provides significant perturbations at the T1 site as discussed (Palmer et al., 1999).

1.6.3 Catalytic Mechanism

Due to the intricacies of laccase, the implicit mechanism has not been fully elucidated, however with the increasing number of crystal structures, spectroscopy, kinetic and theoretical studies, working models have been proposed (Solomon et al., 1996; Lee et al., 2002; Quintanar et al., 2005; Yoon et al., 2007; Huang et al., 1999; Zoppellaro et al., 2001; Shleev et al., 2006). The oxidation of the reducing organic substrate has been described as following a ping-pong type mechanism or a modified ping-pong type mechanism (Shleev et al., 2006; Yaropolov et al., 1994). As previously stated, the reduction of O_2 to H_2O occurs as a result of one-electron oxidation of four molecules of substrate. The substrate interaction is assumed to be similar with the reduction of O_2 to H_2O at the tri-cluster is much more involved than docking of substrate at the T1 site. Therefore substrate docking and oxidation at the T1 Cu site will be discussed first and referred to when discussing the changes in T2/T3 reduction of O_2.

1.6.4 Substrate Docking

As previously stated, the T1 Cu site is the electron acceptor site in which the efficiency of phenols and anilines seem to be correlated to their ΔE° with electron-donor substituents lowering the E° of phenols making them more easily oxidizable by the enzyme. This was confirmed by Xu (1999) (ploting log k_{cat}/K_M vs E°). Kinetic data with the oxidation of substituted phenols using vanadium (v) polyoxotungstate, ie $[\alpha\text{-}SiV^{v}W_{11}O40]^{5-}$, had a redox potential of 0.67 V/NHE but could be used as a model outer-sphere oxidant because these polyoxometalaltes (POM) have redox centers deeply buried within the oxometalate Keggin cage (Galli et al., 2007; Weinstock 1998). These studies found that the POM compound could be used as a legitimate electron transfer model for the electron

transfer oxidation of phenols (Galli et al., 2007). These results also give compelling evidence to support previous kinetic studies in that the outer-sphere electron transfer oxidation is the rate-limiting step because the ΔE° between phenolic substrate (donor) and T1 Cu site (acceptor) dictates the reactivity trend among a series of compounds (Solomon et al., 1999; Lee et al., 2002; Tadesse et al., 2008; Galli et al., 2007). The E° of the substrate is but one factor that must be considered. Structural features of the enzyme also contribute to the catalysis process, including steric issues as well as interactions with amino acids in the local environment. From a crystal structure of *T. versicolor*, a fungal laccase very similar to *T. villosa*, it was found that the negatively charged pocket near the T1 Cu site had hydrophobic residues lining the two sides (Phe 162, Leu 164, Phe 265) and (Phe 332, Phe 337, Pro 391). The entrance to the T1 Cu site is initiated (marked) by two Phe (Phe 332 & 265) ~10.5 Å apart in which the substrate must pass and travel ~6.5 Å before reaching His 458 which is the electron entry point and one of the amino acids ligated to the T1 Cu (Tadasse et al., 2008). Studies with increasingly wider phenols having similar reduction potentials confirmed this and predict a maximum width of < 11 – 12 Å. Substrates with widths larger than this can not be oxidized unless a mediator is used (Tadasse et al., 2008). As the substrate traverses the channel it approaches Asp 206 at the rear of the binding pocket that is deprotonated (pK_a 3.9) at pH's of catalytic relevance (i.e. 5.6-6.0 for this study). The negative charge of the deprotonated Asp 206 attracts -OH or $-NH_2$ to a specific H-bonding interaction positioning the substrate to the His 458 ligand for electron transfer to TI Cu site (Tadasse et al., 2008). When bulky (t-butyl) groups are positioned in the p-position substrate accessibility is not hindered since the bulky substituent is directed outside the binding pocket. It could also contribute to

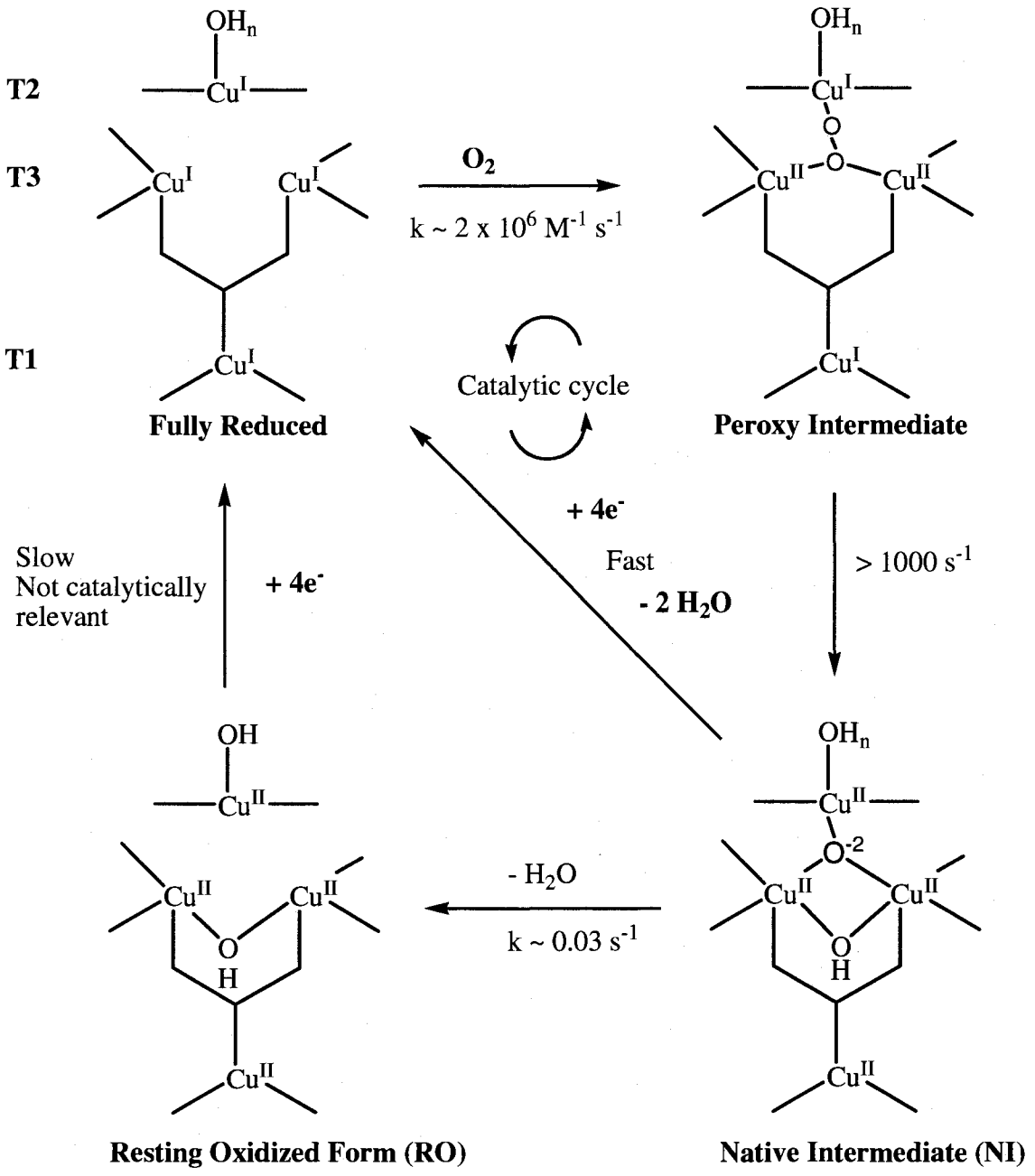
the lower conversion of phenols at low pH's due to Asp being protonated although others have attributed this mainly to the increase in E° of the substrate reducing ΔE° between the T1 site slowing the oxidation rate (Xu 1996; Tadasse et al., 2008; Madzak et al., 2006). Asp 206 was replaced in a site directed mutagenesis study of *T.versicolor* with Glu, Asn, Ala. In comparison of K_M values for 2,6-DMP the values were ordered as follows: Asp ≈ Glu > Ala > Asn. It was postulated that being in the proximity of a carboxylic acid group would likely favour its deprotonation. When a non-phenolic substrate was used the transformation efficiency was altered slightly to: Glu > Asp > Asn > Ala and it was speculated that the radical generated at the T1 site could be stabilized by the electron rich carboxylate side chain and the amide could generate a negative charge on its oxygen atom due to resonance albeit a positive charge generated on the nitrogen. Ala however has no free electrons for radical stabilization (Madzak et al., 2006). The now reduced T1 Cu can shuttle its electrons to the Cu tri-cluster.

1.6.5 Catalytic Cycle

Most of the information discerned about the intermediate and transition states of the Cu tri-cluster is based on spectroscopic, kinetic and theoretical studies of *R. vernicifera* in which E. I. Solomon's group have done extensive research. *R. vernicifera* was selected due to its ideal kinetic and spectroscopic properties (Solomon et al., 1999; Lee et al., 2002). Henceforth the following information is based on these studies and it is inferred that, unlike the T1 Cu site, the T2/T3 Cu site is the same in plant and fungal laccases. Past studies have revealed the presence of two intermediate species termed the peroxy-intermediate and the native intermediate (Cole et al., 1991; Shin et al., 1996; Aasa et al., 1973; Clark and Solomon, 1992) (**Scheme 1.4**). Laccase with a spectrocopically silent,

redox-inactive Hg^{2+} (TIHg laccase), in the TI Cu site has been used to study these intermediates (Palmer et al., 2001; Shin et al., 1996; Quintanar et al., 2005). The generated peroxide bond is then split involving proton and electron transfer that is purely speculative at the time but with an activation energy ~9 kcal/mol which is in close agreement to a one-electron reducing decomposition of peroxide (Palmer et al., 2001). This generates the native intermediate which originally was thought to contain a hydroxyl or oxyl radical, however EPR data indicates that the T2 Cu is coupled to the T3 Cu's (Lee et al., 2002). The NI can slowly transform to the resting oxidized (RO) form of the enzyme with lack of substrate. Both the RO and NI forms of the enzyme have similar redox states but very different structural properties. Its has been proposed that the differences in the two intermediates contributes to the slow decay of the NI to the RO form (Lee et al., 2002; Quintanar et al., 2005; Yoon et al., 2007) (large energy requirement in structural rearrangement). Only one O of the O_2 is found in the RO form of the enzyme, the other is an OH^- (or H_2O) bound to the T2 Cu site, with crystal structure data indicating it is orientated away from the interior of the Cu trimer. In the NI the oxygen atom is bridging the Cu trimer suggesting a large structural rearrangement between the two forms (Lee et al., 2002). The significantly slower turnover in the RO form is attributed to the magnetically isolated, non-bridged T2 Cu, thus under turnover conditions the NI would be the primary acceptor of reducing substrates (or electrons from T1 Site) (Lee et al., 2002). What is interesting in the RO trinuclear cluster is it is highly coordinatively unsaturated and that the positive charge of the oxidized trimer is not compensated by Cu ligation (Quintanar et al., 2005). From **Scheme 1.4** it can be observed that there is an OH^- bridge at the T3 site and an OH^- ligand at the T2 site

leaving a +4 charge on the tri-cluster. The T2 site is a 3-coordinate site to the center of the trimer and the T3 Cu's are four coordinate with trigonal bipyramidal geometry also with open equitoral positions directed to the center of the tri-cluster (Quintanar et al., 2005). The positive charge is stabilized by four conserved Asp/Glu within 12 Å forming a negatively charged pocket as well as the dielectric of the protein matrix. This raises some interesting questions. For example F^- has a high affinity for the tri-cluster due to the insertion of a negative charge decreasing the electrostatic repulsion of the Cu^{2+} ions and overcoming the desolvation energy required for F^- binding to the center of the tri-cluster (Quintanar et al., 2005). Therefore it could be expected that OH^- (H_2O) would also be capable of bridging the tri-cluster, something not observed in the RO form. DFT calculations confirmed that the electrostatic stabilization by the Asp/Glu residues was larger than when the OH^- bound to the tri-cluster by 27 kacal/mol therefore confirming the open coordination positions on the T2 and T3 Cu sites as detected experimentally (Quintanar et al., 2005). These open coordination positions most likely have functional significance promoting the binding of O_2, while the reduction to negatively charged oxo/hydroxo compounds would result in bridged structures with $< + 4$ charge. The electrostatic environment of the pocket destabilizes these bridges and aids in the release of water while inhibiting product inhibition (Quintanar et al., 2005). If the prementioned bridging of OH^- to the RO form did occur it would stabilize the cluster possibly lowering the redox potential out of range for the reduction of O_2 to H_2O (Qunitanar et al., 2005).

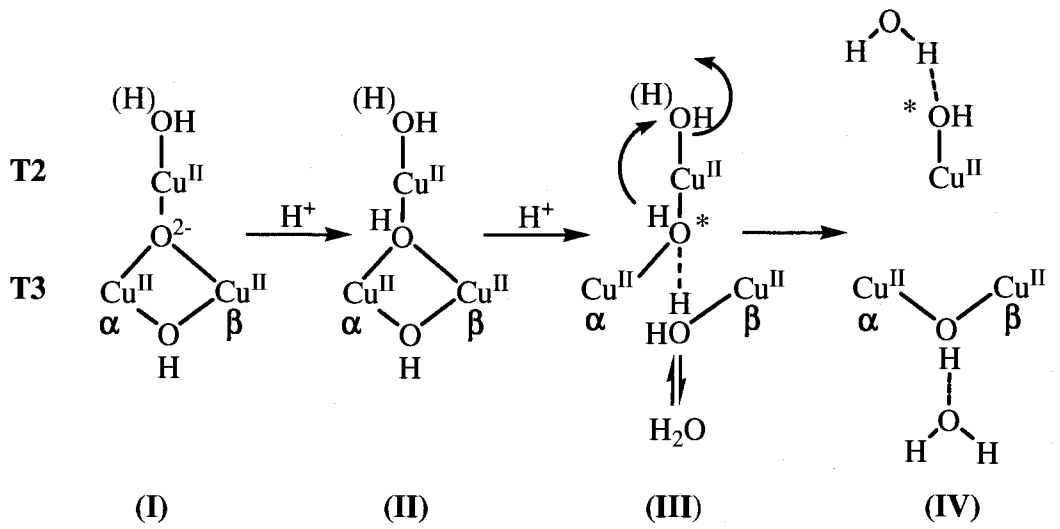


Scheme 1.4: Catalytic Cycle for Laccases adapted from (Lee et al., 2002; Morozova et al., 2007; Yoon et al., 2007)

1.6.6 Native to Resting Oxidized Intermediate

The decay of the NI to the RO intermediate is slow and pH dependent ($t_{1/2}$ = 12-23 s at pH 7.4 vs $t_{1/2}$ = 0.33-1.0 s at pH 4.0-4.2) (Branden & Deinum 1978; Huang et al., 1999).

Recently the comparison of the NI to the NI-azide bound (geometry adapted from crystal structure of *T. versicolor*) model with DFT computations have provided a refinement to the NI structure as well as a possible mechanism for the decay of NI to the RO intermediate (Yoon et al., 2007). This decay involves successive proton-assisted steps with the rate-determining step being the rearrangement of the μ_3 -oxo bridge from the inside to the outside of the tri-cluster as eluded to by Lee et al., 2002 (Yoon et al., 2007) (**Scheme 1.5**). As follows in **Scheme 1.5**, the initial proton binds to the μ_3-oxo center. The second proton goes either to the previously made μ_3-OH or T3 OH- both resulting in the protonation of the T3 OH (due to μ_3-OH proton migration) causing the uncoupling of Cu $T3^{\beta}$ from the other two Cu's. The uncoupled T3-OH ligand could now be interchanged with solvent water. These results have been confirmed since ^{18}O isotope ratio MS (IRMS) has revealed that only one of the O_2 atoms remains (Shin et al., 1996; Branden et al., 1978). The final step involves the reorganization of the O atom on the T2 Cu from the internal side to the external side along with the deprotonation of H_2O if it were ligated to T2 happening as follows: The proton on the H_2O ligand bound to $T3^{\beta}$ transfers to the rotating OH and the reformation of the OH^- bridge between the T3 Cu's is reformed. At this point the now rotating H_2O is externalized following a complete 90° rotation. The final product has the original OH^- or H_2O ligand now replaced by the rotating H_2O with O from the activating O_2 (Yoon et al., 2007). This rearrangement of the NI^{OH} and NI^{H2O} had lower limits for the energy barrier of (8.5 and 18.2 kcal/mol) which are in agreement with experimental results 8.8-13.9 kcal/mol for enthalpy of activation (Huang et al., 1999; Yoon et al., 2007)

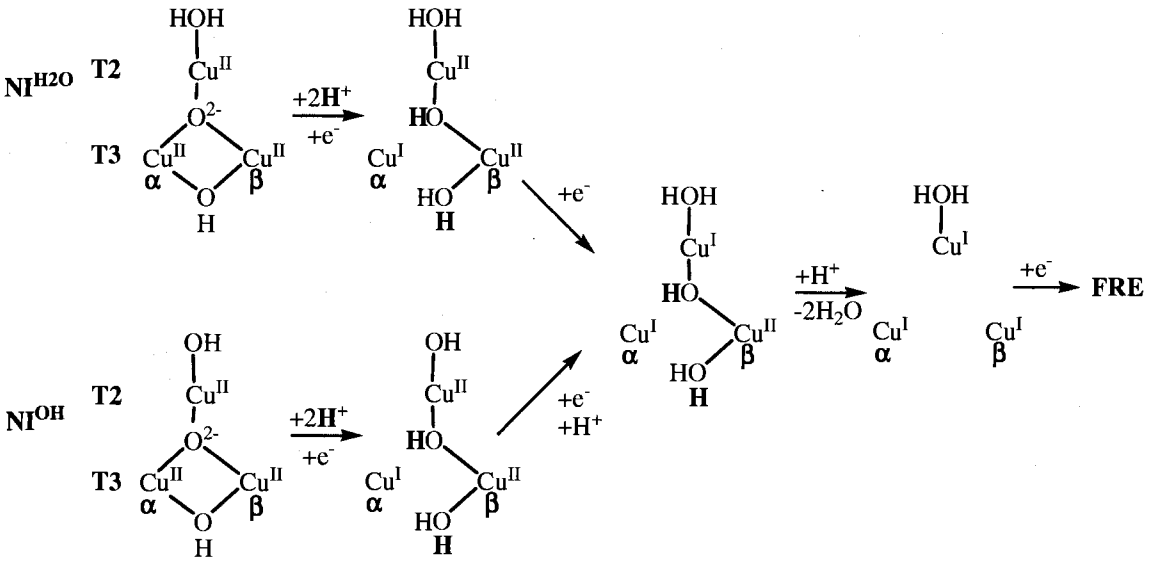


Scheme 1.5: Proposed mechanism for the decay of the native intermediate to the resting oxidized intermediate form of laccase (Yoon et al., 2007). * indicating oxygen initially added for activation. Appears to be rotated 180 in diagram but actually only ~90° facing into the page.

1.6.7 Native intermediate to Fully Reduced

Lastly, the reduction of the NI to the fully reduced form of the enzyme will be reviewed. Both NI and RO forms of the enzyme can be fully reduced although it is believed the sequence in which the Cu's are reduced varies (Lee et al., 2002; Huang et al., 1999; Zoppellaro, 2001). **Scheme 1.6** demonstrates a proposed mechanism for the 4 e^- reduction of the NI to the fully reduced form of laccase. **Scheme 1.7** highlights the suspected variations in the order in which the Cu's are reduced for both the NI and RO forms. **Scheme 1.6** shows the sequential reduction of the tri-cluster via oxidation of reducing substrate at the T1 Cu site for both the NI^{OH} and NI^{H2O} intermediates. The Cu $T3^{\alpha}$ is the first since it was found to be lower in energy than that of the T2 or $T3^{\beta}$ which both have a lower redox potential because of an Asp residue in the vicinity. The Asp residue is thought to have little effect on the NI form due to all the Cu's being oxidized (Yoon et al., 2007). Since the T3 Cu's are ligated to three His the reduction of $T3^{\alpha}$ and

the protonation of the oxo center will most likely be accompanied by protonation of the OH^- bridge as it dissociates from the reduced $T3^{\alpha}$. Next the T2 Cu is reduced, in the NI^{OH} form this reduction is most likely to follow or be coupled to the protonation of the T2 ligand raising the reduction potential of the T2 site (Yoon et al., 2007). The still coupled $T3^{\beta}$ Cu and T2 Cu by OH^- allows for the rapid transport of electrons from the T1 Cu all the way to the T2 Cu. If the $T3^{\beta}$ Cu is reduced first this OH^- bridge will be broken and severely hinder the reduction of the T2. Notice in **Scheme 1.7**, the reduction of the RO form which is much slower in returning to the fully reduced form already has a decoupled T2 Cu, as well it is proposed that both the T3 Cu's are reduced before the T2 Cu (Lee et al., 2002). The reduction of the T2 Cu before the $T3^{\beta}$ would produce a mixed valence Cu pair which spectroscopic studies have confirmed (Reinhammer, 1981; Reinhammer et al., 1980). The final step would be the reduction of $T3^{\beta}$ which would be quick due to the Cys-His pathway as well as the protonation of the OH- T2-$T3^{\beta}$ bridge releasing two H_2O molecules (Yoon et al., 2007). Lastly, the reduction of the T1 Cu site to produce a laccase with a complement of reduced Cu's (the FRE).



Scheme 1.6: Possible mechanism for the reduction of NI to the fully reduced enzyme (FRE) (Yoon et al., 2007)

Reduction of Resting Oxidized (Slow)

T2	Cu^{II}	Cu^{II}	Cu^{II}	Cu^{II}	Cu^{I}
T3	Cu^{II}_{α}--Cu^{II}_{β}	$\xrightarrow{e^-}$ Cu^{II}_{α}--Cu^{II}_{β}	$\xrightarrow{e^-}$ Cu^{I}_{α} Cu^{I}_{β}	$\xrightarrow{e^-}$ Cu^{I}_{α} Cu^{I}_{β}	$\xrightarrow{e^-}$ Cu^{I}_{α} Cu^{I}_{β}
T1	Cu^{II}	Cu^{I}	Cu^{II}	Cu^{I}	Cu^{I}

Reduction of Native Intermediate (Fast)

T2	Cu^{II}	Cu^{II}	Cu^{I}	Cu^{I}	Cu^{I}
T3	Cu^{II}_{α}--Cu^{II}_{β}	$\xrightarrow{e^-}$ Cu^{I}_{α} Cu^{II}_{β}	$\xrightarrow{e^-}$ Cu^{I}_{α} Cu^{II}_{β}	$\xrightarrow{e^-}$ Cu^{I}_{α} Cu^{I}_{β}	$\xrightarrow{e^-}$ Cu^{I}_{α} Cu^{I}_{β}
T1	Cu^{II}	Cu^{II}	Cu^{II}	Cu^{II}	Cu^{I}

Scheme 1.7: The possible order of reduction of the Cu's from NI and RO form to the Fully Reduced Enzyme (FRE), dashed lines represent bridging (Yoon et al., 2007; Lee et al., 2002).

Scheme 1.7 shows the difference in the order of Cu reduction to the two intermediate forms of the enzyme. The reduction of the native intermediate has already been discussed including a possible mechanism for electron proton transfer (**Scheme 1.6**). The reduction of the RO intermediate is based upon results with Cp (Ceruloplasmin) in which the second electron along with the reduced T1 is involved in a two electron reduction of the T3 Cu atoms (Machonkin and Solomon, 2000). The T2 Cu was also the last to be reduced but this is supported by the lack of T2-T3 bridging impeding a quick ET (Machonkin and Solomon, 2000; Lee et al., 2002). It was originally proposed that a two-electron transfer from the T1 combined with the second electron from substrate being transferred to the T2, $T3^{\alpha}$ Cu producing the mixed valance binuclear T3 site but the model was modified to fit the mechanism proposed by Yoon et al., 2007.

1.7 Additives

Early studies with HRP attributed the high enzyme requirements to inactivation due to the phenoxy radical attacking the enzyme's active site (Klibanov et al., 1983). Since the major disadvantage of enzyme-based treatments is the cost of the enzyme, anything to reduce inactivation would concurrently reduce the amount of enzyme and cost of treatment regime making it more competitive. Nakamoto and Machida (1992) speculated that the end-products may adsorb some of the active enzyme and hinder substrate from reaching the active site. Therefore they proposed that the addition of other proteins or hydrophilic synthetic polymers would decrease inactivation. This provided direct evidence that the enzyme's inactivation is not solely due to radical attack on the active site. In the original study a gelatine mixture, polyvinyl alcohol (PVA), milk casein, bovine serum albumin and PEG's of various MW all had a suppressive effect on enzyme

inactivation. It was determined that PEG with MW above a thousand was the best additive. It was proposed that adsorption based on competitive H-bonding with additive suppressing enzyme inactivation by leaving more free enzyme in solution (Nakamoto and Machida, 1992). Since then a number of enzyme-pollutant-additive combinations have been investigated with both peroxidases and laccases summarized in **Table 1.5**. However the exact mechanism in which this protective effect is provided is still not known as well as the type of interaction that may be involved.

Table 1.5a: Some of the Reported "PEG Effects" with Peroxidases						
Enzyme	**Substrate**	**No PEG (U/mL)**	**PEG (U/mL)**	**% Removal**	**[PEG] mg/L**	**Ratio[a]**
Peroxidases						
SBP[(1)]	Phenol	0.90	0.60	> 95	50	1.50
SBP[(1)]	o-Chlorophenol	0.23	0.19	> 95	40	1.21
SBP[(1)]	m-Chlorophenol	0.65	0.15	> 95	75	4.30
SBP[(1)]	p-Chlorophenol	0.20	0.15	> 95	30	1.33
SBP[(1)]	o-Cresol	0.60	0.08	> 95	400	7.50
SBP[(1)]	m-Cresol	0.75	0.08	> 95	150	9.38
SBP[(1)]	p-Cresol	0.60	0.40	> 95	20 – 40	1.50
SBP[(1)]	2,4-dichlorophenol	0.08	0.04	> 95	150	2.00
SBP[(1)]	Bisphenol A[b]	0.90	0.015	> 95	60[b]	60
SBP[(2)]	Phenol	1.35	0.32	> 95	35*	4.2
ARP[(3)]	Phenol	6.0	0.4	> 95	100[†]	15
ARP[(3)]	o-Cresol	10.0	2.0	> 95	100[†]	5
ARP[(3)]	m-Cresol	6.0	0.2	> 95	100[†]	30
ARP[(3)]	p-Cresol	2.0	0.4	> 95	100[†]	5
ARP[(3)]	o-Chlorophenol	10.0	0.1	> 95	100[†]	100
ARP[(3)]	m-Chlorophenol	10.0	0.2	> 95	100[†]	50
ARP[(3)]	p-Chlorphenol	10.0	0.05	> 95	100[†]	200
ARP[(3)]	2,4-dichlorophenol	6.0	0.02	> 95	100[†]	300
HRP[(7)]	Phenol	2.0	0.05	> 95	30	40
HRP [(7)]	Phenol[e]	30.0	0.4	> 95	250	75
HRP[(4)]	o-Cresol	3.0	0.10	> 95	10	30
HRP[(4)]	m-Cresol	2.8	0.03	> 95	30	93
HRP[(4)]	p-Cresol	1.0	0.03	85	30	33
HRP[(4)]	o-Chlorophenol	3.0	0.03	> 95	100	100
HRP[(4)]	m-Chlorophenol	5.0	0.50	> 95	100	10
HRP[(4)]	p-Chlorophenol	2.0	0.015	> 95	30	132
HRP[(4)]	2,4-dichlorophneol	0.5	0.015	> 95	10	33
HRP[(5)]	Phenol[c]	4.5	0.3	100		15
Coprinus[(6)] UAMH 10067	Phenol[c]	0.75	0.6	> 95	200[†]	1.25
Coprinus[(6)] UAMH 4103	Phenol[c]	0.9	0.6	> 95	200[†]	1.5
Purified[(6)] Coprinus	Phenol[c]	13.5	0.6	> 95	200[†]	22.5
Tomato hair root [(9)]	Phenol[d]	Only run under stringent conditions: Basic Peroxidase, and Ionically Bound Peroxidase purified fractions) were 3 to 13 times higher then when PEG absent leading to higher conversion				
Turnip Peroxidase [(8)]	Phenol, 2-chlorophenol, 3-chlorophenol, BPA, 2,4-dichlorophenol, o-cresol, m-cresol[b]	PEG (100-200mg/L) was shown to reduce reaction time to obtain > 95% removal of initial substrate. Up to 230% increase in remaing TP activity was reported. Strict optimization for individual substrates and PEG concentration was not done.				

1.0 mM substrate using PEG_{3350} unless stated, run for 3 h. at optimum pH determined in individual studies (6.0-8.0 for SBP, 7.5 for ARP, *Coprinus* 7.0); [(1)] Caza et al., 1999; [(2)] Kinsely and Nicell, 2000 * PEG 35 000; [(3)] K.E. Taylor et al., 1996; [(4)] Wu Y. et al., 1993); [(5)] Ikehata and Buchanan, 2002; [(6)] Ikehata et al., 2005; [(7)] Wu J. et al. 1993 [(8)] Duarte Vazquez et al., 2002; [(9)] Gonzalez et al., 2008; [a] [E] no PEG/[E] with PEG; [b] 0.5 mM; [c] 1.1 mM; [d] 0.10 mM; [e] 10.0 mM [†] PEG Concentration not optimized.

Table 1.5b: Some of the Reported "PEG Effects" with Laccases						
Enzyme	**Substrate**	**No PEG (U/mL)**	**PEG (U/mL)**	**% Removal**	**[PEG] mg/L**	**Ratio[a]**
T. versicolor[(1)]	BPA [b]	0.45	0.30	> 95	43	1.5
T. villosa (SP 504) [(2)]	BPA	0.0109	0.0021	> 95	147	5.2
T. villosa (SP 504) [(3)]	Diphenylamine [c]	.0075	.0035	> 95	80	2.1
T. villosa (SP 504)[(4)]	2,4-dimethylphenol	~0.008	~0.004	> 95	1-4	2.0

1.0 mM substrate using PEG_{3350} unless stated, run for 3 h. at optimum pH determined in individual studies Laccase all between pH 5.0-6.0; [(1)] Kim and Nicell, 2006; [(2)] Modaressi et al., 2005; [(3)] Saha et al., 2006; [(4)] Ghosh et al., 2008; [a] [E] no PEG/[E] with PEG; [b] 0.120 mM; [c] 0.19 mM

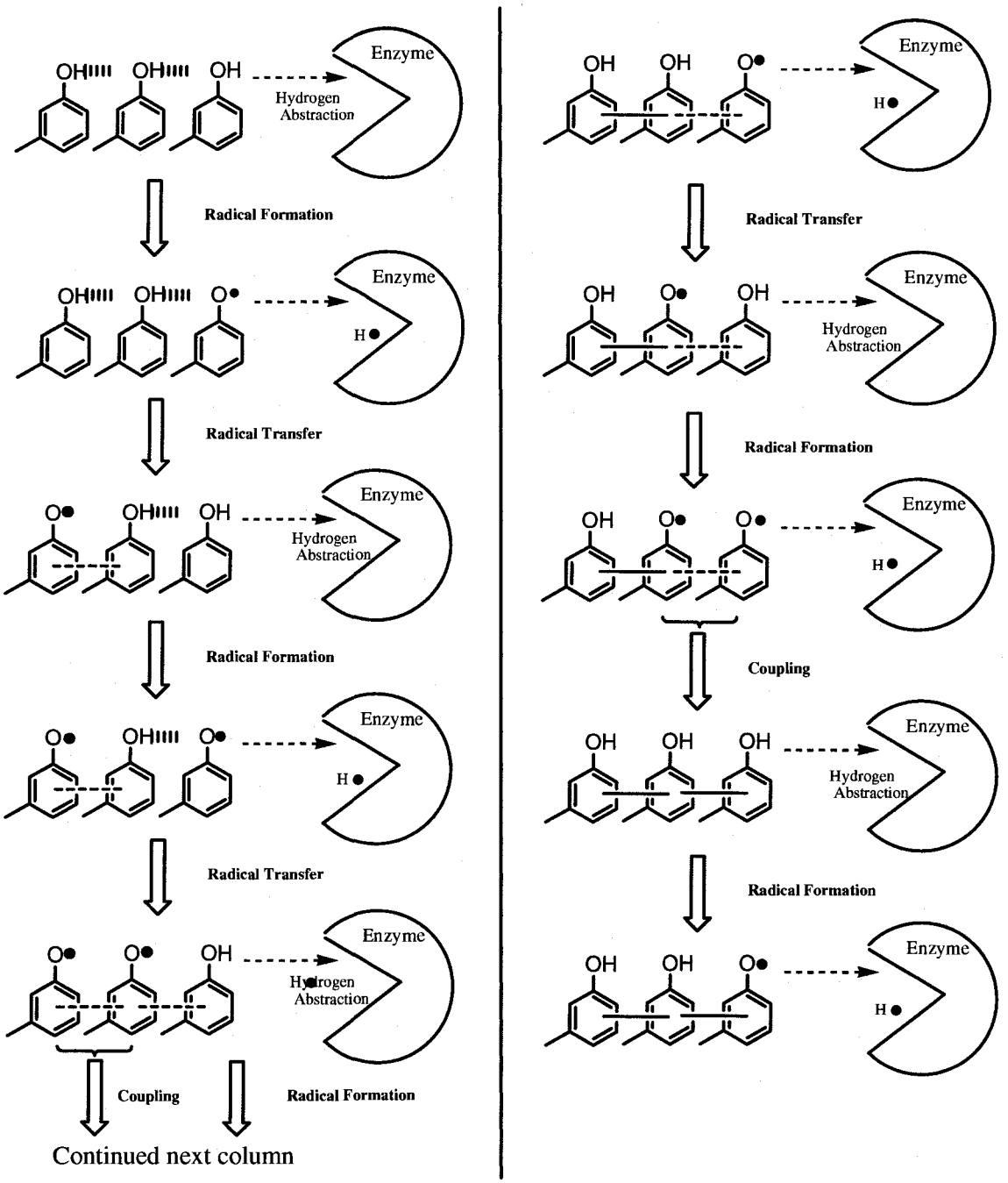
The additives that have been effective with either laccase or peroxidase do not seem to have any similarities in relation to their monomeric chemical structure. The more effective polymers tend to be non-ionic and have the capacity to form globular structures. In numerous accounts, PEG below a MW of 1000 (400, 300) did not protect the enzyme in the case of peroxidases (Kinsely and Nicell, 2000; Wu et al., 1998; Nakamoto and Machida, 1992) or only provided an intermediate protective effect with laccase (Kim and Nicell, 2006). It was also demonstrated that when polymeric products were isolated from reactors and re-incubated with fresh enzyme that they still had the capacity to deactivate the enzyme despite no H_2O_2 being present, while polymeric products made with PEG in similar experiments did not inactivate the enzyme (Wu et al., 1998). Some kinetic evidence with laccase suggests that the increase in efficiency is due solely to prolonged enzyme activity since initial oxygen consumption was not increased while total oxygen consumption over the reaction period was increased (Kulys et al., 2003). It should also be noted that the only non-phenol compound observed to have this "PEG-effect" is the secondary amine diphenylamine (model compound for dyes) with laccase (Saha 2006). This would suggest that the phenolic products/intermediates have a higher affinity for the hydrophilic synthetic and natural polymers. It still has not been determined in its entirety

what the affiliation the polymers have with the enzyme, intermediates, or products during a reaction. It has also been reported that the appearance of products made with PEG tend to be lighter in colour than when the additive is absent (Wu Y. et al., 1998). Little work has been done on the identification of products after enzymatic treatment let alone in the presence of additives (Huang and Weber, 2005). When comparing crude and purified enzyme the purified enzyme is more susceptible to inactivation and has larger "PEG effects" than the crude fractions most likely due to other proteins or stabilizers having a similar protective effect acting as sacrificial polymers, taking away from part of the observed PEG effect with purified enzyme. It is usually observed that crude fractions as opposed to purified enzyme work better when no additives based on the pre-mentioned reasoning however, few studies where the comparison of residual activity over the course of the reactionr were done with crude and purified samples with and without additive (Ikehata et al., 2005). This would be consistent with Nakamoto and Machida's original model for protecting the enzyme.

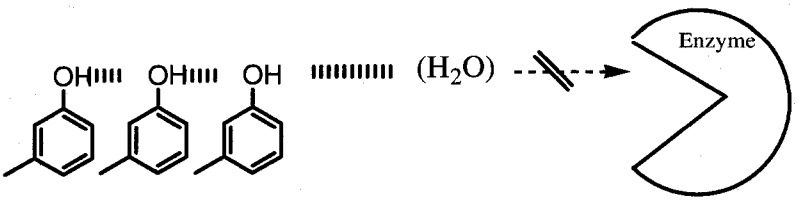
Another possible reason for using additives is as templates for radical polymerization (Polowinski 2002). In recent years laccases and peroxidases have been receiving a lot of attention for their ability to catalyze the oxidative polymerization of phenols to synthesize a new class of polyphenols with remarkable thermal stability and without the use of formaldehyde that conventional phenolic resin preparation requires (Oguchi et al., 2002). These reactions are run in various mixtures of aqueous organic solvents. The aqueous phase (buffer) was used so that the enzyme retained some of its activity, and the organic solvent was used to help solubilize the generated products so that larger polymers were formed (Oguchi et al., 2002). Solvent and buffer ratio greatly affect the MW of the

polymers synthesized however the solvent effect or the influence of hydrophobic interactions are hard to explain by macroscopic solvent properties, such as dielectric constant (Oguchi et al., 2002). In a study of m-cresol polymerization by HRP in methanol-buffer and 1,4-dioxane-buffer mixtures it was determined that the maximum MW of the polymeric product was found at significantly different organic:solvent ratios (50% , 20% aqueous content, respectively) (Oguchi et al., 2002). UV studies of m-cresol and methanol-water and m-cresol and 1,4-dioxane-buffer mixtures demonstrated a decrease in absorbance at 283 nm in which the decrease was monotonic with the increase in buffer content. Mass spectrometry results correlated the initial increase UV absorbtion at 285 nm to m-cresol clustering and the decrease in absorbtion at 270-278 nm and 280-290 nm to the increased hydration of these clusters. Thus it was concluded that the clustering effect is controlled by the microscopic structure (solvation) around m-cresol but not desolvation (Oguchi et al., 2002). The polymerization of m-cresol is shown in **Scheme 1.8** in which the polymerization mechanism varies slightly from that proposed in **Scheme 1.1** and explains why an increase in water content affects the MW of polymer that **Scheme 1.1** would not account for. In **Scheme 1.8** a cluster of m-cresol is H-bonded together (although some distortion expected). The enzyme then produces the generally accepted phenoxy radical that should be continuously generated through the radical transfer process. If radical transfer and formation occur faster in the cluster form, it would provide a more efficient pathway for producing polymeric product. It would also explain why increasing buffer concentration yields polymers with lower MW. When the hydration of the cluster is sufficiently high the cluster's interaction with the enzyme is shielded as seen in **Scheme 1.9**. This could explain why the MW of polyphenol product

was substantially lower in 100 % aqueous solution; the lack of organic solvent would also help facilitate product precipitation. It also explains why methanol-buffer mixtures are more affected by water content since m-cresol is preferentially solvated by dioxane than water, while m-cresol will interact directly with both methanol and water (Oguchi et al., 2002). With this in mind PEG as a template for reactions run in 100% aqueous solutions were investigated with HRP (Kim Y.-J. et al., 2004; Kim Y.-J. et al., 2003, Oguchi et al., 2002). . Templates were used not only to help cluster the monomer of substrate closer togther but also as a way of controlling the regeoselctivity (coupling selectivity) in an attempt to synthesize more uniform polymers. Template radical polymerization can follow one of two main mechanisms; zip (**Scheme 1.10**) and pick-up (**Scheme 1.11**) (**Polowinski 2002**). PEG with phenol and PEGMDE (poly-(ethylene glycol) monododecyl ether) cause increase in specific peaks around 277 and 282 nm presumed to be due to the formation of a H-bonded complex with PEG or PEGMDE based on the pre-stated results by Oguchi and coworkers (Kim Y.-J. et al., 2004; Kim Y.-J. et al., 2003, Oguchi et al., 2002). In both cases, the increase in absorbance intensity increased and then leveled off after phenol:PEG or PEGMDE was close to a 1 :1 ratio (with respect to oxyethylene repeating units in the polymeric additive) suggesting the interaction between phenol and PEG/PEGMDE through a zip mechanism (Kim Y.-J. et al., 2003; Kim Y.-J. et al., 2004).

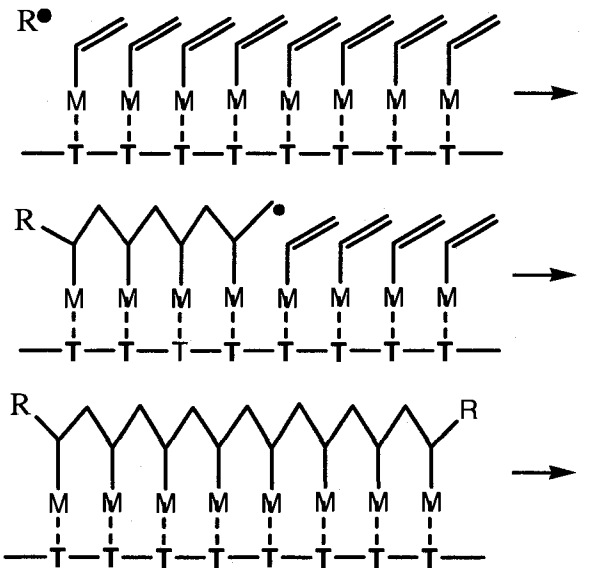


Scheme 1.8: Possible mechanism to polyphenol synthesis in organic aqueous solution using peroxidases. Reproduced from Oguchi et al., 2002.

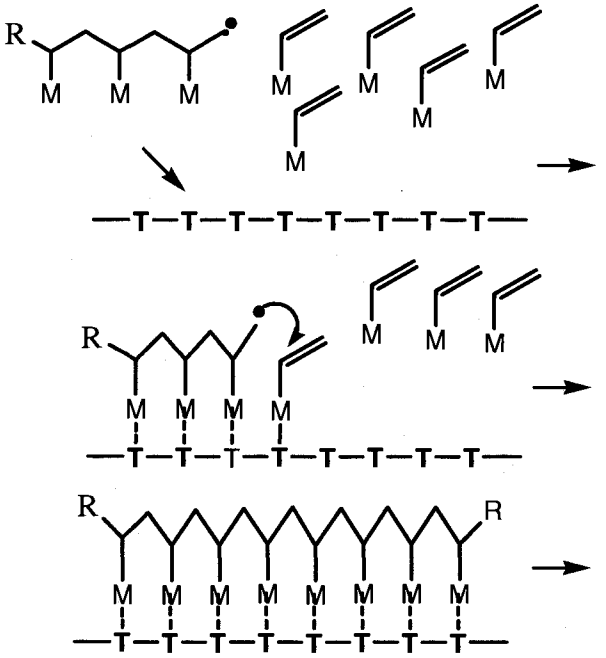


Scheme 1.9: Hydration of clusters; leading to low MW polyphenols. Reproduced from Oguchi et al. (2002).

Interestingly, increases in PEG MW greatly affected the yield of the reaction with peroxidase. When the degree of polymerization (DP) of the additive was > 13 the yield was high (polymeric product) and decreased dramatically when DP was < 9 (Kim Y.-J. et al., 2003). It is thought that under these conditions the phenol-PEGMDE complex is not formed adequately and hinders efficient polymer production. It was also determined that under these conditions products had phenylene unit contents approaching 90% and MW's in the range 3700-4900, which was affected little by the PEG chain length used as a template. In methanol-buffer solution phenylene unit contents were 40-70% suggesting that template polymerization may have better control over regioselectivity than changing the hydrophobicity of monomer and solvent. Phenylene unit content did increase with template length (PEG) suggesting that hydrophobicity of the template may have some effect on regioselectivity. The FT-IR spectrum as well as H-NMR confirmed that PEG was associated in the product most probably by H-bonding interactions (Kim Y.-J. et al., 2004). Laccase has also been used for polyphenol synthesis in methanol buffer organic aqueous mixtures producing similar phenylene/oxyphenylene ratios to HRP of 48:52 with almost identical FT-IR spectrum but the aid of a template in 100 % aqueous solution has not been documented to our knowledge (Mita et al., 2003).



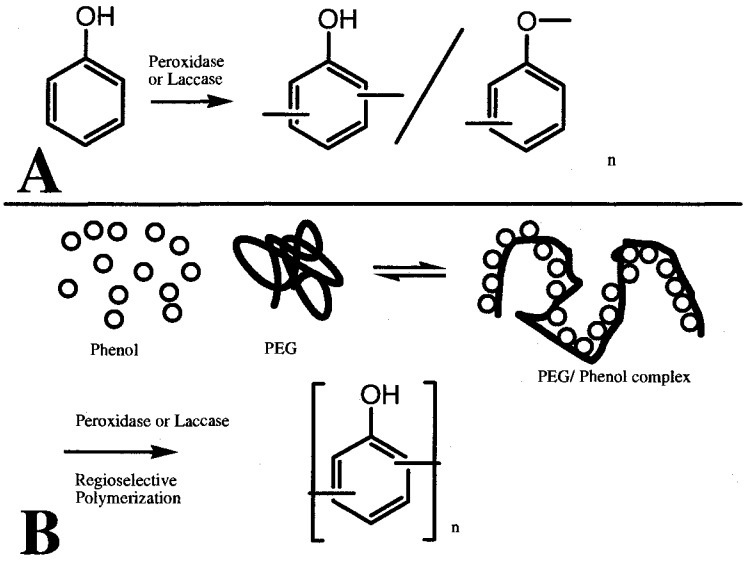
Scheme 1.10: Schematic representation of chain template polymerization type I (Zip mechanism). Reproduced from Polowinski (2002).



Scheme 1.11: Schematic representation of chain template polymerization type II (Pick-up mechanism). Reproduced from Polowinski (2002).

Scheme 1.12 demonstrates the proposed model for PEG and phenols by Kim Y.-J. and co-workers (2003). In this model PEG works as a type 1 template (zip mechanism) for radical polymerization. As seen in **Scheme 1.12,** when the additive is not added the

reaction produces a mixture of phenylene and oxyphenylene units but when the template is added the regioselctivity is better regulated producing a more uniform polymer with mainly phenylene units.



Scheme 1.12: PEG utilized in a zip mechanism for synthesis of a more uniform polyphenol. Reproduced from Kim Y.-J. et al., 2003.

Some of the other additives that have reduced requirements for enzyme concentration have also been used as template polymers for polymerization such as PVP, and chitosan (Polowinski 2002). However reactor conditions such as temperature, template concentration and solvent have to be considered.

Chapter 2: Objectives

The objectives are subdivided into four sections

Part I: Optimization for Conversion of Substrates

1. Feasibility of using laccase SP 504 from *Trametes villosa* to catalyze the oxidative polymerization of phenols (phenol, o-,m-,p-cresol) and anilines (aniline, o-,m-,p-toluidine) in synthetic wastewater batch reactors.
2. Determine optimum operating parameters to achieve ≥ 95 % conversion of phenols and anilines in a 3 h reaction period, including effect of pH, enzyme concentration.
3. Effectiveness of additives, including PEG, PVP, dextran , PEI , and Triton X-100, in reducing minimum laccase concentration for ≥ 95 % conversion of o-, m-and p-cresols .
4. Determine the minimum effective PEG concentration for various substrate concentrations.
5. Determine if there is a trend to enzyme concentrations required for ≥ 95 % conversion with and without PEG for various initial substrate concentrations of o- , m-, and p-cresols.
6. Examine the effect of PEG MW (400-35,000) as an additive in laccase-catalyzed conversion of o-, m-, and p-cresols.
7. Determine the fate of PEG in reactors.
8. Determine how much enzyme activity is lost through the 3 h reaction period and how the addition of PEG affects this.

9. Determine the effect that reducing anions, halides and cyanide of various concentrations that are commonly found in industrial effluents may have on the enzyme.

Part II: Kinetics

1. Determine how PEG as an additive may affect kinetic parameters of o-,m-,p-cresol and how it may pertain to the PEG effect

Part III: Refinery Wastewater

1. Determine the feasibility of using laccase SP 504 in the removal of phenol from authentic wastewater and how it compares to synthetic samples
2. Optimize parameters, including pH, enzyme concentration and PEG, for batch reactors in an attempt to remove ≥ 95 % of the phenol in a 3 h reaction period.
3. Investigate the influence of reducing anions, halides and cyanide on catalytic oxidative polymerization of phenol.

Part IV: Preliminary Product Identification

1. Seek NMR evidence of mixtures that PEG still associated with products confirming TOC results
2. Assign chemical characteristics to products made with and without PEG.

Chapter 3: Materials and Experimental Protocol

3.1 Materials

Enzyme: Laccase SP 504 *(Trametes villosa,* a fungal source, and laccase SP 850 (source?) were developmental preparations made by Novozymes North America, Inc (Franklinton, NC), obtained as gifts. Laccase SP 504 using Batch US-1999-000091-03 and US-1999-000091-04 both with nominal activity of 200 LACU/g were stored in the freezer at-15°C until stock solutions were required to be made. Laccase SP 850 from Batch # US Enz-142, Nominal activity of 1170 LAMU/mL was also stored at -15°C until needed. **Substrates** were of analytical grade of 99% purity or greater and include: o, m, p-cresols, o, m, p-toluidines and phenol crystals all purchased from Aldrich Chemical Co. (Milwaukee WI). Aniline was obtained from BDH Inc. (Toronto ON). **Colorimetric assay** reagents 4-aminoantipyrine (4-AAP), potassium ferricyanide, 2,4,6-trinitrobenzene sulfonic acid (TNBS) (1.0 M in H_2O stored at -15°C), and syringaldazine (stored 2-8°C in a desiccator) were purchased from Sigma Chemical Co. (St. Louis MO). **PEG's / Additives:** PEG_{400}, PEG_{1450}, PEG_{8000}, and PEG_{3350} were purchased from Sigma Chemical Co. (St. Louis MO), while PEG_{35000} was obtained from Fluka Chemie (GmbH CH-9471 Buchs Switzerland) Polyvinylpyrolidine with average MW's of 10,000 (PVP-10) and 40,000 (PVP-40) as well as dextrans produced by *Leuronostoc mesenteroides* strain N. B-519, industrial grade with average MW of 80,000 and 282,000 were also purchased from Sigma Chemical Co. (St. Louis MO). Polyethylenimine (50% wt % solution in H_2O) (PEI) with average MW of 750,000 and octylphenoxy polyethoxyethanol (Triton X-100 avaerage of 9 or 10 EO units) were purchased from Aldrich Chemical Co., Milwaukee,

WI. **Reducing agents and halides** were made from their sodium salt forms of ≥ 99% purity: sodium thiosulfate, sodium sulphate, sodium nitrate, sodium cyanide, sodium fluoride, sodium chloride, sodium bromide crystals, sodium iodide crystals were purchased from BDH Inc. (Toronto ON). **Buffers and solvents:** glacial acetic acid, sodium acetate, monobasic sodium phosphate, concentrated hydrochloric acid, sulphuric acid and 95% ethanol were purchased from ACP Chemicals Inc. (Montreal QC). (2-[N-Morpholino]ethanesulfonic acid) monohydrate (MES) > 99.5 % purity with pH range from 5.5-6.7 and Tris(hydroxymethyl)-aminomethane > 99.8% purity was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium borate, boric acid crystals, HPLC grade acetonitrile and methanol were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Dimethyl sulfoxide-D6 (D, 99.9%) used for NMR analysis was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA). **Metal standards** for atomic absorption Cd, Cu, Pb, and Zn were made from EnviroConcentrate™ (10,000 µg/ml) in dilute HNO_3 obtained from ULTRA Scientific (North Kingstown, RI). A stock solution of Fe was made from 1025 µg/mL in 1 wt. % HCl stock solution (purchased from Aldrich Chemical Co., Milwaukee, WI). Mg was made from $MgCl_2$ in 1 wt. % HCl. **TOC:** Potassium hydrogen phthalate, sodium hydrogen carbonate, sodium carbonate were purchased from Sigma Chemical Co. (St. Louis MO) **Others:** Acrodisc® 25 mm syringe filters with 0.2 µm HT tuffryn membranes, non-sterile for low protein binding and Accrodic® 13 mm LC13 PVDF syringefilters with 0.2 µm polyvinylidine fluoride (PVDF) membranes .were purchased from Pall Gelman Laboratories (Mississauga ON); nitrocellulose membrane filters. 0.45 µm, white gridded sterile filters from Millipore Corporation (Bedford MA). EM Quant test strips for cyanide (semi quantitative)

purchased from EM Science (Gibbstown, NJ). Aluminum sulphate (alum) was purchased from BDH Inc. (Toronto ON). Dialysis Tubing from Spectrum®Labs, Spectra/Por® 1, regenerated cellulose (RC), molecular weight cut off (MWCO) 6000-8000. The five refinery wastewater samples were donated by a confidential source.

All other chemicals used in the following studies were of analytical grade and purchased from BDH Inc. (Toronto ON.) or Aldrich Chemical Corporation (Milwaukee, WI).

Methods

3.2 Analytical Methods

3.2.1 Buffer Preparation

A variety of buffers was used depending on the application and desired pH. Buffers of various concentration and pH were made according to Gomori (1955): Acetic acid-sodium acetate buffers for pH 4.0-5.8, monobasic-dibasic sodium phosphate buffer for pH 5.6-8.0, boric acid sodium borate buffer for pH 7.6-9.2, and MES buffer for pH of 5.5. pH was confirmed using Orion Research Inc. 420A pH meter or I.Q. Scientific Instruments Inc. EA940 pH meter with a stainless steel micro pH probe (PH15-SS) obtained from London Scientific (London, ON).

3.2.2 Phenol Colour Test

Phenolic substrates were quantified using a colorimetric based assay developed as a standard method (Faust & Mikulewicz et al 1967). The electrophilic attack of the phenolic substrate by 4-AAP produces a leuco compound that is then oxidized to a quinone-type compound by potassium ferricyanide with a λ_{max} in the visible range at

approximately 510 nm (**Appendix A**). All developing times were within 15 min and stable for at least 1 h. p-Cresol was the only compound that did not react in the colorimetric assay and therefore it was only analyzed by HPLC.

3.2.3 TNBS Test

This is a simple and accurate colorimetric assay for analyzing amines and aniline compounds. In this study it was used exclusively on the anilino type compounds, aniline, o,m,p- toluidine. Due to a long colour development time and low molar extinction coefficient in comparison with the other three compounds, o-toluidine was only analyzed by HPLC. When the pH of the reaction mixture is above the pKa of the aniline compounds the reactions proceed in a nucleophilic aromatic substitution of TNBS with the formation of a tetrasubstituted ring carbon intermediate. Sodium sulfite was also added so that a more stable sulfite mono adduct of TNBS is formed with a higher λ_{max} of approximately 420 nm (Wang et al., 2005; Means et al., 1972). See **Appendix B** for more detailed description.

3.2.4 Laccase Activity Test

All activity tests for SP 504 were run at pH 5.5 and have been calculated in the units of activity designated LACU/mL by Felby 1998. All activity tests for laccase SP 805 were run at pH 7.4 and have been calculated in the designated units of LAMU/mL also designated by Felby 1998. One laccase unit is the quantity of enzyme required to catalyze the conversion of 1 μmole of syringaldazine per minute. The catalytic oxidation of syringaldazine is monitored by the increase in absorbance at 530 nm which is proportional to the generation of its corresponding quinone (**Appendix C**).

3.2.5 Cyanide and Sulfide Test Strips

Refinery samples were tested for cyanide with EM Quant Test Strips with semi-quantitative detection range from 0-30mg/L. The barbituric acids contained in the strips would produce a red polymethine dye when reacting with glutaconic dialdehyde. Glutaconic dialdehyde formation occurs when cyanide ions react with chlorine to form cyanogen chloride causing ring opening of pyridine resulting in glutaconic dialdehyde. All samples were pH adjusted to within the effective testing range between pH 6-7 with dilute sulfuric acid. Strips were stored at 20-25°C.

Sulfide in refinery samples was tested for using lead acetate test strips. The sulfide reacts with lead acetate to form lead sulfide (grey black in colour). The detection limit is 5.0 mg/L. Strips were stored at 20-25°C.

3.3 Analytical Equipment

3.3.1 UV-VIS Spectroscopy

Absorbances were measured with a model 8452A Hewlett Packard Diode Array Spectrometer equipped with Vectra ES/12 computer (λ range of 190-820 nm and 2.0 nm resolution). Quartz cuvettes with 1.0 cm pathlength were purchased from Hellma (Concord, ON). Substrate direct absorbance standard curves can be found in **Appendix E.**

3.3.2 HPLC Analysis

All substrates were also quantified using a Waters HPLC System with 1525 binary pumps, 2487 Dual λ absorbance detector, 717 Plus autosampler, equipped with Symmetry C_{18} (5μm, 4.6 x 150mm) column and operated by Breeze 3.2 or 3.3 software.

Phenol, o-, m-, p-cresols were run under isocratic conditions with 60% acetonitrile and 40% aqueous with 0.1% acetic acid for synthetic samples. When refinery wastewater samples were being analyzed phenol analysis was done with 40% acetonitrile and 60% aqueous 0.1% acetic acid. Aniline, o-, m-, p-toluidines were run under isocratic conditions with 50% methanol and 50% ≤ 20 mM phosphate buffer at pH (7.0-7.4) (Rigorous purging with water was done after to remove any precipitating buffer after each sample set). Flow rate was 1.0 mL/min; injection volumes of 10μL; column was not heated. The UV-detector was adjusted according to the predetermined λ_{max}'s. Calibration curves are found in **Appendix D**.

Table 3.1: HPLC Conditions

Aromatic Substrate	**Mobile Phase Solvents**		**Ratio of Solvent in Mobile Phase as %**		**Wavelength Detector Set at (nm)**
	Pump A	**Pump B**	**Pump A**	**Pump B**	
o-Cresol	Acetonitrile	Acetic Acid aq (0.1%)	60	40	272
m-Cresol			60	40	272
p-Cresol			60	40	278
Phenol			60	40	270
Phenol			40	60	270
Aniline	Methanol	20mM Phosphate Buffer aq (7.4)	50	50	280
o-Toluidine			50	50	280
m-Toluidine			50	50	284
p-Toluidine			50	50	288

3.3.3 Total Organic Carbon (TOC) Analysis

Aqueous PEG after reactions was determined by TOC using a Shimadzu TOC-V CSH Total Carbon Analyzer purchased from Shimadzu Scientific Instruments (Columbia, MD) using oxygen as the oxidizing agent. Calibrations for total carbon (TC) and inorganic carbon (IC) were done using 2125 mg/L of hydrogen phthalate and a combination of 3500 mg/L of hydrogen carbonate and 4410 mg/L of sodium carbonate, respectively.

TOC was determined by the subtraction of IC from TC (TOC = TC-IC). Inorganic carbon is the structural basis for inorganic compounds such as gas carbonates and carbonate ions. The IC of the sample is defined as the dissolved CO_2 and carbonates dissolved in the water. The samples are acidified with HCl (or phosphoric acid) to a pH below three converting all carbonates to CO_2. These samples are volatilized by bubbling N_2 through the sample that contains no CO_2. TC is measured by the combustion of the sample in a combustion tube with an oxidation catalyst and heated to 680 - 700 °C. TC is converted to CO_2 in which a carrier gas (N_2) at a flow rate of 150 mL/min carries the products to an electronic dehumidifier where the gas is cooled and dehydrated. Samples are then carried through a halogen scrubber to remove chlorine and other halogens. Finally the combustion products are analyzed by a non-dispersive infrared (NDIR) gas analyzer to quantify CO_2. All reaction samples were micro-filtered prior to making the 100 µL sample injection to avoid clogging the machines lines with the insoluble polymers generated through enzymatic treatment.

3.3.4 Atomic Absorption Spectroscopy

Metals were analyzed with a Shimadzu 6800 series atomic absorption spectrometer equipped with an ASC-6100 autosampler. The lamp current was 5 mA, oxidant was air and acetylene was used as fuel for all samples. **Table 3.2** Summarizes slit width and recommended detection concentrations for each metal tested.

Table 3.2: Atomic Absorption Settings			
Metal	**Wavelength (nm)**	**Optimum Analytical Range (µg/mL)**	**Slit Width (nm)**
Cd	324.7	0.03-1.0	0.5
Co	240.7	0.05-15.0	0.2
Cu	324.7	0.03-10.0	0.5
Fe	248.3	0.06-15.0	0.2
Mg	285.2	0.05-5.0	0.5
Pb	217.0	0.10-30.0	1
Zn	213.9	0.01-2.0	1

3.3.5 Nuclear Magnetic Resonance (NMR)

Enzymatic product was dissolved in deuterated DMSO (1 mg/mL) and microfiltered with LC prep syringe filters to ensure all non-dissolved solids were removed. ^{1}H-NMR was run on a Bruker DPX 300 MHz NMR instrument with broadband AX Probe.

3.3.6 Additional Equipment:

pH Meters: Orion Research Inc. USA, 420A pH meter or I.Q. Scientific Instruments Inc. EA940 pH meter was equipped with a stainless steel micro pH probe (PH15-SS, 211, 205,201) obtained from London Scientific (London, ON). Calibration buffers (pH 4.00, 7.00, 10.00) were obtained from BDH Inc. (Toronto ON.).

Magnetic Stir-plates: Micro V magnetic stirrers (0-1100 rpm, model 4805-00) and VWR MAGSTIRRER (100-1500 rpm, model 82026-764) were purchased from VWR International Inc. (Mississauga, ON.). Magnetic Stir bars supplied by Cole-Parmer Chicago, IL.).

Microfilters and Plastic Syringes: 0.2 µm HT Tuffryn membrane syringe filters 25 mm diameter, low protein binding, were purchased from Pall Gelman Laboratory

(Mississauga ON.). Plastic syringes obtained from Becton Dickinson Corporation (Clifton NJ.).

Centrifugation was done with a Beckman J2-HS floor model, refrigerated centrifuge, with maximum speed of 21,000 rpm. All samples for these studies spun at $\leq$ 8000 rpm.

3.4 Experimental Protocol

Laccase Catalyzed Polymerization of Phenols and Anilines:

All reactions were run in duplicate or triplicate at ~ 20 - 25°C in 30 mL batch reactors. Synthetic wastewater was comprised of a buffered solution containing a single substrate, phenol, o, m, p-cresol, aniline or o, m, p-toluidine, ranging in concentration from 0.25 mM to 10.0 mM in a total volume of 20 mL. Laccase was added last to initiate the reaction which was run for 3 h, an arbitrary time that has been used in our lab for comparison purposes. In reactor design the retention or contact time may need to be optimized for operational cost. If you require certain retention times the enzyme concentration can be optimized for such conditions by increasing enzyme concentration for shorter contact times and reducing concentrations for longer contact times. . All batch reactors were stirred on magnetic stirrers and open. 1800 µL samples were quenched using 200 µL of 0.5 M H_2SO_4 to adjust the pH below 2, thereby denaturing the enzyme and halting substrate conversion. Samples were then microfiltered and analyzed by the appropriate colorimetric assay and HPLC with the exception of p-cresol and o-toluidine which were only analyzed by HPLC.

pH Optimization with and without Polyethylene Glycol: Batch reactors with synthetic wastewater samples containing 1.0 mM of the pre-mentioned substrates were run under stringent conditions (where enzyme was the limiting factor in conversion) and 40 mM

buffers. Identical series with PEG_{3350} present at 100 mg/L were run at the same time with identical buffers, and buffer concentration. pH was altered from ~2.0 – 10.0 where pH <3.0 was adjusted with dilute H_2SO_4; acetate buffer was used for pH's 4.0-5.8, phosphate buffer from 5.6-8.0, borate buffer for pH's 7.6-9.2 and dilute NaOH for pHs > 9.2. Enzyme concentrations were varied depending on substrate and if PEG_{3350} was present. Samples were then quenched, microfiltered and analyzed via colorimetric assay HPLC or both. Solution pH was examined before and after the reaction was carried out to confirm that pH did not differ by more then ± 0.05 pH units and that the buffer was not compromised.

Optimum Enzyme Concentration for ≥95% Substrate Conversion with and without Polyethylene Glycol: Batch reactors with synthetic wastewater samples in 40 mM buffer were run at the optimum pH for the substrate being investigated, with increasing laccase concentrations until ≥ 95% conversion of initial substrate was observed. Substrate concentration of cresols ranged from 0.25 mM - 3.0 mM while phenol, aniline and o-,m-, and p-toluidine were only run at 1.0 mM. For studies involving PEG_{3350} a slight excess was added to ensure that enzyme was the limiting factor (50 – 100 mg/L /mM).

Polyethylene Glycol as an Additive: Batch reactors with synthetic wastewater samples comprised of 40 mM phosphate buffer, containing substrate concentrations of 1.0-5.0 mM for o,m,p-cresol while phenol, o, m, p-toluidine were only run at only 1.0 mM, PEG_{3350} ranging from 0-400 mg/L were run with Laccase concentration being limiting to significant conversion for 3 h (laccase concentrations found in Results). After 3 h, samples were acid quenched, microfiltered and analyzed for remaining substrate. Some

substrates were also analyzed at additional time intervals of 18 and 24 h to confirm the previous assumption that PEG has a protective effect.

Effect of Polyethylene Glycol MW: Batch reactors with synthetic wastewater containing 1.0 mM o,m, or p-cresol, 40 mM phosphate buffer of 5.8 pH, and PEGs ranging from 0 – 400 mg/L under stringent conditions with laccase concentration being the limiting factor to significant conversion. PEG's with average MW's of 400, 1450, 3350, 8000, and 35,000 were used and reactors were run for 3 h before being acid quenched, microfiltered and analysed via colorimetric assay. Note for all preceding studies, only PEG_{3350} was used unless otherwise stated.

Effectiveness of Other Additives: Batch reactors with synthetic wastewater containing 1.0 mM o-, m-, p-cresol, 40 mM phosphate buffer of pH 5.8, and PVP with average MW's of 10,000 and 40,000, dextran with average MW's of 80,000 and 282,000, PEI with average MW of 750,000, and Triton X-100 were added in two series, one in 5 mg/L concentration and another in 50 mg/L with laccase being limiting to significant substrate conversion. Reactors were acid quenched after 3 h, microfiltered and analyzed by HPLC.

Fate of Excess Polyethylene Glycol: Batch Reactors with synthetic wastewater made up of 40 mM phosphate buffer with initial substrate concentrations of 1.0 and 3.0 mM for p, m- cresol and 1.0 & 5.0 mM for o-cresol with excess enzyme to ensure 100% conversion. Reactions were run for 3 h and any samples with cresol remaining received another aliquot of enzyme or were left overnight until substrate was converted. Only the 5.0 o-cresol reactors had significant o-cresol remaining (see Results). The entire 20 mL reactor content was acidified, microfiltered and then analyzed for remaining TOC. TOC for PEG was calculated by taking the total carbon (TC) and subtracting the inorganic carbon (IC)

as well as the TOC for any residual cresol detected by HPLC. Some soluble product remained in solution but because the conversion for all batch reactors were similar increasing TOC as PEG concentration increases was assumed to be solely due to residual PEG in supernatant and not increased solubility of products.

Activity and Removal Over Time: Batch reactors were set up with synthetic wastewater containing PEG when applicable, 40 mM phosphate buffer at pH 5.6-5.8 and 1.0 mM substrate, with a total volume of 50 mL and various laccase concentrations depending on desired conversion and substrate under investigation. Samples were acid quenched every 15 or 30 min, microfiltered, and analyzed by HPLC. Activity was also monitored over time by quantifying the laccase activity as a % of the activity in a controlled batch reactor with no substrate present. Activity tests for some substrates required serial dilutions in order to meet the requirements for the Laccase Activity Assay as specified in **Appendix C**. Micro-filtered and non-micro-filtered samples were both subjected to the activity tests. A similar protocol was used when running studies with laccase SP 850 except optimum pH for those reactors were run using phosphate buffer at pH 7.0, in accordance to previous optimization by Vermette (2000).

Kinetics: Initial velocity for the conversion of o,m,p-cresols with and without PEG_{3350} were monitored by keeping the laccase concentration the same and varying substrate concentration until adequate data was obtained to estimate single substrate kinetic parameters (K_M and V_{max}). 50 or 100 mL batch reactors were run with 40 mM phosphate buffer at optimum pH for each substrate and slight excess to predetermined optimum PEG mg/L:mM ratios which were kept constant for all trials. Samples were acid-quenched at 0, 2, 4, 6, 8 and 10 min, microfiltered and analyzed by HPLC. All reactors

were aerated ahead of time and it was assumed that within the first 10 min O_2 would be saturating. m-Cresol with PEG was not run due to solubility issues in higher concentrations. When keeping the PEG:substrate ratio constant, PEG was not sufficiently soluble in the higher substrate concentration ranges leaving an incomplete data set for analysis.

Reducing Anions and Halides: Reducing anions and halides were introduced to synthetic wastewater made from their sodium salt forms at a concentration of 200 mg/L (based on anhydrous weight) in the batch reactor. Thus reducing anions from sodium thiosulphate (1.27 mM), sodium sulphate (1.41 mM), sodium nitrate (2.35 mM), sodium cyanide (4.08 mM) as well as halides from sodium fluoride (4.76 mM), sodium chloride (3.41 mM), sodium bromide (1.94 mM) and sodium iodide (1.33 mM) were used. Reactions of 20 mL volumes of 1.0 mM phenol or p-cresol in 40 mM phosphate buffer were run for three hours against a control with no reducing anions or halides under stringent conditions for three hours. Samples were then acid quenched, microfiltered and analyzed by HPLC. To further investigate the effect of cyanide, increasing concentrations were run for three hours followed by acid quenching, micro-filtration and HPLC analysis. p-Cresol removal over time in the presence of reducing anions was done as stated in previous time course studies.

Minimum Alum Concentration: Reactors for synthetic phenol were run under the predetermined optimum conditions, pH 5.6-6.0 in 40.0 mM phosphate buffer, 0.08 LACU/mL, in which significant polymeric products were generated that did not settle on their own (brown in colour with visible precipitate). Increasing alum concentrations were added, 0.0-2.2 mM, followed by pH adjustment to 7-10 with NaOH for gel formation.

Similar studies were done with refinery samples except that initial pH was adjusted down to the optimum pH with H_2SO_4 and not buffered.

Refinery Wastewater: Samples were stored at room temperature (20-25°C) with initial phenol and pH recorded. All experiments were done within one month of receiving the samples. A slight decrease in pH was observed over this time period.

Preliminary Product Identification: o-Cresol oxidative polymerization by laccase SP 504 in 500 or 50 mL batch reactors with 10 mM o-cresol, 10-40 mM phosphate buffer, 100-400 mg/L PEG (excess) when applicable and excess enzyme. Dialysis of laccase SP 504 was done (due to an initial finding of PEG proton peaks in the non-PEG reactors) using Spectr/Por® 1 dialysis tubing and 40 mM phosphate buffer with three washes at room temperature for 1-1.5 hrs each and then 2 washes in the fridge. TOC of the washes was done to confirm removal of any organic carbon (could be PEG or soluble products). Products were then resuspended in H_2O and centrifuged for 10 – 15 min at 8000 rpm. Three washes were collected and also analyzed by TOC. The products produced with PEG as an additive present and absent were noticeably different. Both sets of products were dissolved in DMSO (1.0 mg/mL) and ^1H- NMR was taken to confirm previous TOC results.

3.5 Sources of Error

All experiments are susceptible to systematic and random errors that affect the accuracy and reliability of results. All samples had standards run every 10 to 15 samples to ensure accuracy and performance of machines. Graphs show averaged values of a minimum of duplicate or more trials. Error bars for standard deviation were excluded when below 4.0%. When multiple sample sets were run on the same day standard deviation was

always below 3.0% excluding the enzyme activity assay. When samples were run several days error approached a maximum of 8.0% attributed to several factors not strictly controlled such as temperature, stirring level, loss of enzyme activity. Laccase activity had only a finite lifetime and a loss of activity was observed due to freeze thawing over the years. Stock solutions of laccase in buffer only had a lifetime of 3 days before a noticeable decrease in activity, therefore nominal values based on activity of concentrated solutions (200 LACU/mL) were reported and corrected based on the decay over time. Activity assays were reproduced within a maximum deviation of 10.0-15.0 %, when run on multiple days, thought to be due to inaccurate pipetting of the crude stock solutions as well as large dilution factors required. Standards were frequently remeasured in order to assess the validity of values and analytical methods.

Chapter 4: Results and Discussion

4.1 pH Optimization

Enzymes are proteins with dynamic polymeric structures containing characteristic ionizable side chains of amino acid residues responsible for structural and catalytic functions. pH changes can cause perturbations in structural geometry by altering the charge state of these ionizable side chains, denaturing the enzyme, or diminishing its catalytic activity. Previous studies involving fungal laccases suggest that pH-related structural or mechanistic changes can affect the enzyme, as well as the difference in substrate redox potential in comparison to T1 Cu, and oxygen binding at the T2/T3 Cu tri-cluster (Xu 1997). Fungal laccases tend to have a spectral acid-base transition between pH 6 and 9 around the T1 copper site and optimal catalytic activity in acidic pH's between 4.0 and 6.0, but are generally stable in the pH range 3-9. (Xu 1997; Xu 1996; Modaressi et al., 2005; Ghosh et al., 2008; Saha, 2006; Kim and Nicell, 2006). Fungal laccases generally have biphasic bell-shaped pH curves with phenolic substrates which is attributed to the opposing effect of redox potential at the T1 Cu site which favours increasing pH for phenolic substrates and the binding of hydroxide anions to the T2/T3 Cu tri-cluster prohibiting the binding of molecular oxygen at higher pH (Tadasse et al., 2008; Xu 1997). Oxidation of substrates such as ABTS and $K_4Fe(CN)_6$ showed monotonic pH profiles with rate of conversion (activity) decreasing as pH increased supporting the fact that hydroxide anions are inhibitory. The oxidation of ABTS to cation radical (more stable) or $K_4Fe(CN)_6$ to $K_3Fe(CN)_6$ do not involve the transfer of protons making E° independent of pH, supporting the previous hypothesis. Due to the recent availability of crystallographic studies two key amino acid residues interacting

with the reducing substrate have been identified, His(458) and Asp(206) in *Trametes versicolor* conserved among fungal laccases (Madzak et al., 2006; Tadasse et al., 2008). His(458) also coordinates the T1 Cu which is the primary electron acceptor (Madzak et al., 2006). Asp(206) is located in the rear of the binding pocket surrounded by hydrophobic residues thought to position the substrate. It has been observed that an increase in pH from 3 to 5 usually decreases the K_M of substrate and since phenolic substrates are all protonated at this pH (pK_a's 9.9- 10.2) deprotonation of the Asp(206) (pK_a 3.9) conferring a negative charge is speculated to be the source of attracting substrates with -OH or $-NH_2$ functionality to participate in a specific hydrogen bonding interaction (Tadesse et al., 2008; Madzak et al., 2006; Betrand et al., 2002; Bukh et al., 2006). This helps orient the substrate for electron transfer to His(458) to the T1 Cu (Tadesse et al., 2008; Bukh et al., 2006).

Optimum pH is defined as the pH in which the enzyme has optimum catalytic efficiency based on the amount of substrate converted. The effect of pH on the conversion of various reducing substrates, at 1.0 mM, was studied in the pH range 2-10, under "stressed" ("stringent") conditions, i.e.- where enzyme concentration was the limiting factor resulting in incomplete removal within a 3 h reaction period, excess PEG was used when applicable. Studies were originally done on phenolic compounds (phenol, o,m,p-cresols) with and without PEG. The corresponding anilines (aniline, o,m,p-toluidines) were later done to compare the effect of ring functionality. Optimum pH ranges were the range in which conversion was within 5.0% of the maximum observed conversion for the series based on the average of triplicate batch reactors for the phenolics and duplicates for the anilines (**Table 4.1**, page 75). Optimum pH ranges for the phenolics can be observed

in **Fig. 4.1-4** of which all are within the acidic pH range 5.1-6.6. It should be noted that this may not be the optimum pH for treating the wastewater, in that a balance between the capitol and operating cost will have to be considered. If the cost of altering the effluent was considerable then in some cases it may be beneficial to run the reactor at a pH wich is not optimum for the enzyme but still effective enough for reasonable conversion. This balance between operational and capital cost must be considered for all optimized parameters in reactor to treatment strategies. Conditions of high catalytic stability usually decrease retention times thus reducing reactor volume and saving cost. Of the four phenolic substrates, the three cresol isomers were observed to have "PEG effects" (discussed later) and with the addition of PEG o- and p-cresols became less sensitive to acidic pHs (**Fig. 4.2,4.4**). A slight suppression effect is observed when borate buffer was used with o-, m-cresol. Borate/boron has shown suppression effects with peroxidase in the past (Nakamoto and Machida, 1992)

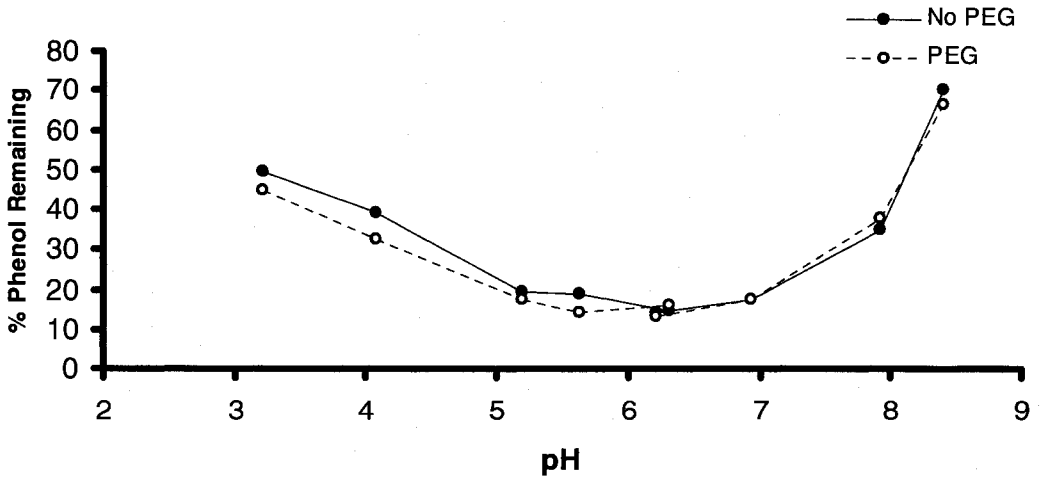


Figure 4.1: pH Optimization for Phenol with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM phenol; 0.0625 LACU/mL and 100 mg/L PEG when required)

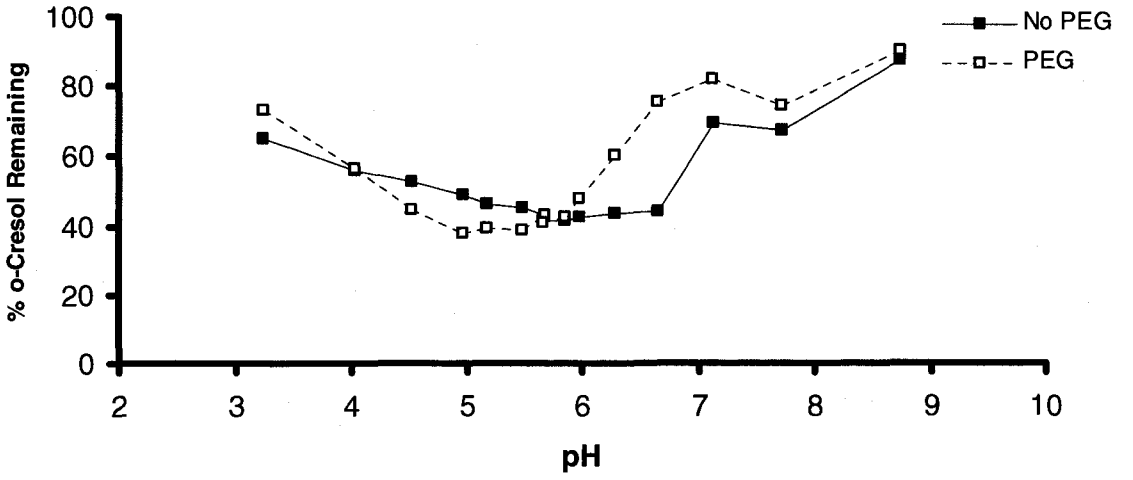


Figure 4.2: pH Optimization for o-Cresol with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM o-cresol; 0.015 and 0.0015 LACU/mL without and with PEG, respectively, and 100 mg/L PEG when required; analyzed by colour test only)

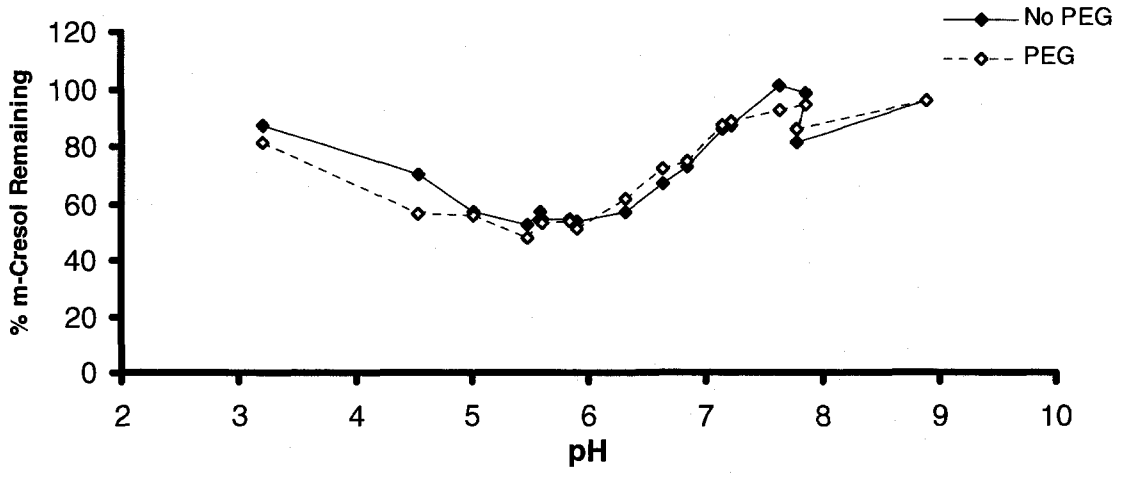


Figure 4.3: pH Optimization for m-Cresol with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM m-cresol; 0.01 and 0.005 LACU/mL without and with PEG, respectively, and 100 mg/L PEG when required)

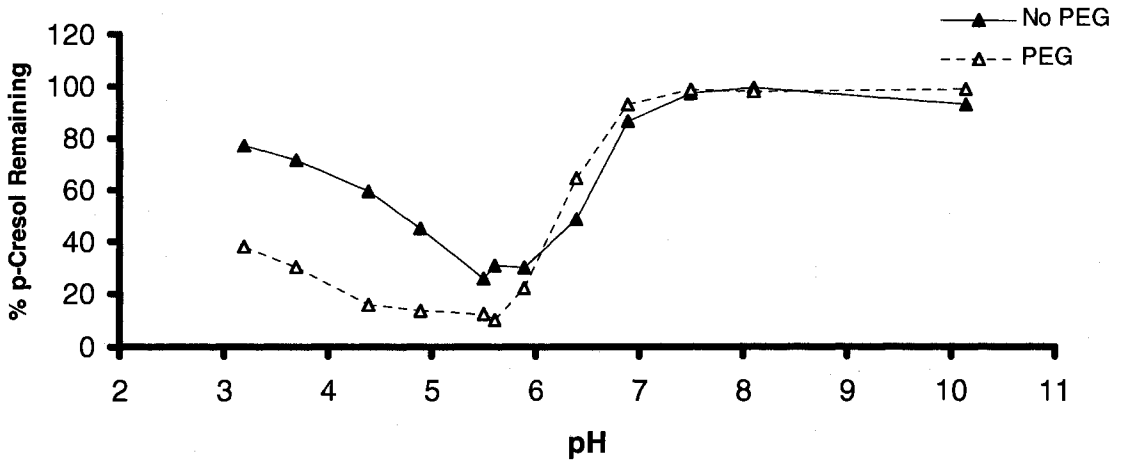


Figure 4.4: pH Optimization for p-Cresol with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM p-cresol; 0.003 and 0.0015 LACU/mL without and with PEG, respectively, and 100 mg/L PEG when required)

Replacing the -OH groups from the phenolics with $-NH_2$ groups: aniline, o,m,p-toluidines were run under the same conditions previously stated for the corresponding phenolic compounds. The anilines showed very similar optimum pH ranges, again on the acidic side within 4.7-6.4, slightly more acidic than the phenols (**Table 4.1 Fig. 4.5-8**). Samples were analyzed by colorimetric assay and HPLC. The colorimetric assay should react with most free amino groups attched to an aromatic ring (TNBS slow with substituents in o-position, phenol colour test inhibited by substituent in para-position) while HPLC should be able to differentiate between these species and authentic substrate. It is expected when using any colorimetric test that those values to be slightly higher then HPLC results because they will react with some of the soluble products. No significant deviations in pH optimization were observed by the addition of PEG to the aniline compounds.

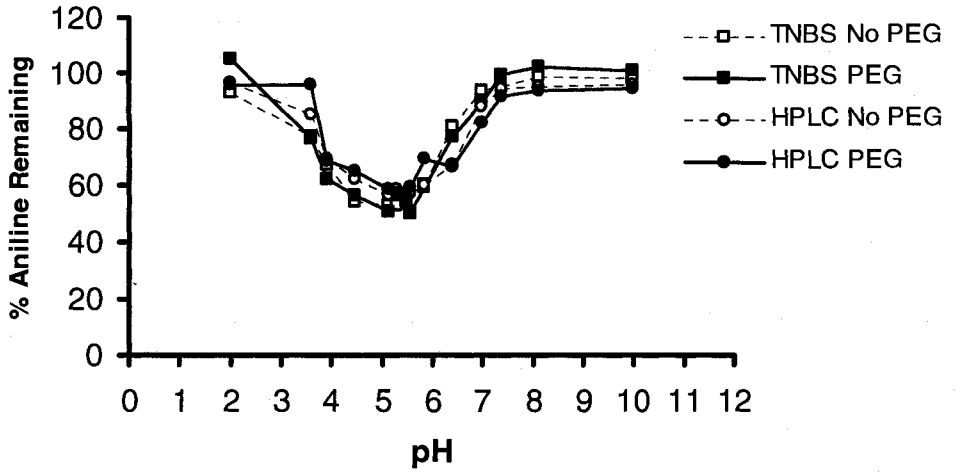


Figure 4.5: pH Optimization for Aniline with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM aniline; 0.2 LACU/mL with and without PEG and 200 mg/L PEG when required; analysis of remaining anilines was carried out in two ways: colorimetrically using TNBS, chromatographically by HPLC)

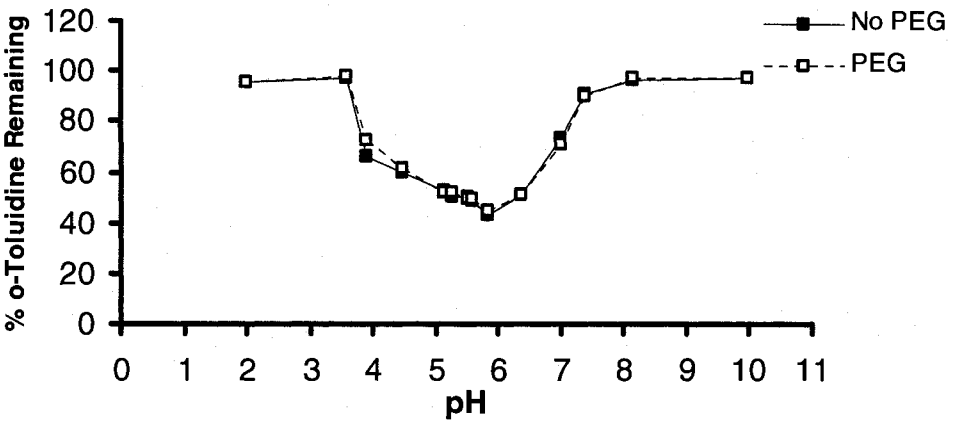


Figure 4.6: pH Optimization for o-Toluidine with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM o-toluidine; 0.05 LACU/mL with and without PEG, and 200 mg/L PEG when required)

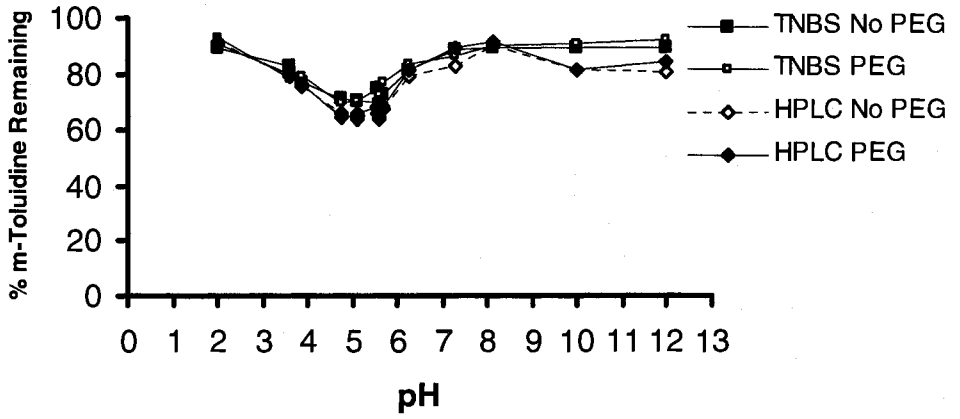


Figure 4.7: pH Optimization for m-Toluidine with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM m-toluidine, 0.05 LACU/mL with and without PEG, and 200 mg/L PEG when required)

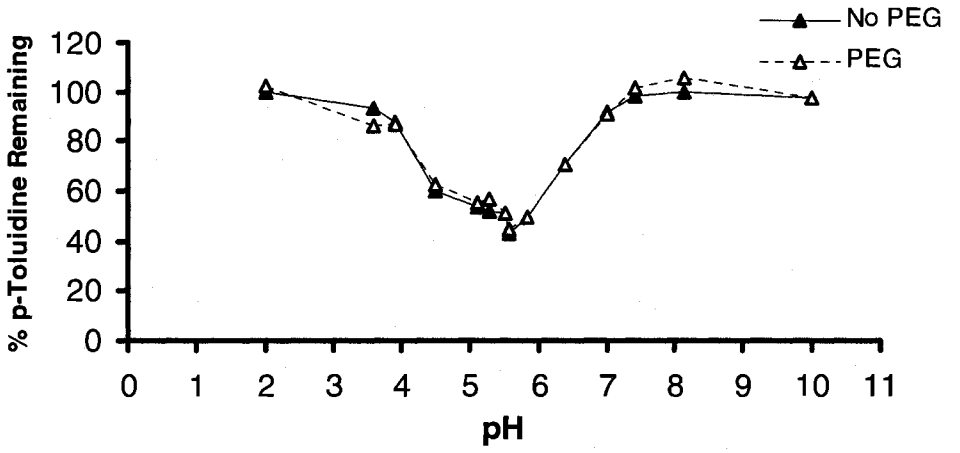


Figure 4.8: pH Optimization for p-Toluidine with and without PEG. (3 h reaction; 50 mM buffers; 1.0 mM p-toluidine; 0.025 LACU/mL with and without PEG, and 200 mg/L PEG when required, only TNBS test)

Since no significant shift in the optimum pH range was observed by altering the functional group and location and despite the fact that the pK_a of the phenolic and aniline compounds vary greatly (4.63 aniline - 10.2 o-cresol) it is likely that the optimum pH is based primarily on the ionization state of laccase rather than substrate (pKa referring, here and throughout this dissertation to the conjugate acid of the aniline compounds ;

pointing out that optimum catalytic efficiency is when the respective compounds are primarily in their neutral state). The inverted bell-shaped pH curves are consistent with previously reported results and can be explained by the pre-mentioned opposing effects caused by an increase in pH. In a study by Xu (1997) comparing three fungal laccases (*Trametes villosa, Rhizocyonia solani, Myceliopthora thermophilia*) and three dimethoxyphenols with pK_a's ranging from 7.0 to 8.7, each laccase showed a distinct optimum pH range that was independent of the others as well as substrate. The same was found in this study. Some speculation on these results can be made based on the redox potentials of the T1 Cu site and the T2/T3 Cu site as follows: With increasing pH from 2.7 to 11.0, the redox potential, E°, of phenol would decrease by 0.49 V at 25°C (Xu 1997). Over the same pH range, the redox potentials of the Cu in the T1 Cu site of *Trametes villosa, Rhizoctonia solani, Myceliophthora thermophilia,* and *Rhus vericinifera* showed a decrease of ≤ 0.1 V (Xu 1997).

$\Delta E° = E°(\text{T1 Cu}) - E°(\text{substrate})$ **[1]** (Xu 1997)

$\Delta E° = E°(\text{T2/T3 Cu's}) - E°(\text{T1 Cu})$ **[2]** (Xu 2001, Xu et al., 1998))

$\Delta E° = E°(O_2/H_2O) - E°(\text{T2/T3 Cu's})$ **[3]** (Xu 1997)

Equation 1 would indicate that pH increases would increase $\Delta E°$ and facilitate faster electron transfer reducing the T1 Cu^{+2} to Cu^{+1} translating into increased rate of conversion of substrate (Kurniawati and Nicell, 2008; Xu 1997). Equation 2 shows that an increase in pH would have the opposite effect with hydroxide ion binding (or any anion) to the T2/T3 tri-cluster reducing $\Delta E°$ and slowing the internal electron transfer possibly to the point where it becomes the rate limiting step (Xu 1997, Xu 1998, Malmstrom 1997). Equation 3 illustrates how the change in O_2 reduction potential due to

pH could also be a factor: for example, E° of (O_2/H_2O) decreases from 1.23V to 0.58V from pH 0.0–11.0 at 20°C which would result in a decrease in $\Delta E°$ and hence slower reaction rate at higher pH's (Kurniawati and Nicell, 2008; Xu 1997). Therefore Equation 1 supports the increase in conversion from the increase in pH while the sharp decrease in conversion as pH is increased is supported by equations 2 and 3. Similar results using the same laccase as in this study with BPA (pH 5.4-6 no PEG 4-6 with PEG) and 2,4-DMP (pH 5.6 no PEG 5.2 with PEG) were observed, as well as the decreased sensitivity in the acidic range (Ghosh et al., 2008; Modaressi et al., 2005). Diphenylamine had a PEG effect of 2.0 at 0.19 mM initial substrate (Saha 2006). No inference into the lack of pH sensitivity can be made based upon the addition of PEG at this time.

Table 4.1: Optimum pH Range With and Without PEG

Substrate	Optimum pH Range	
	No PEG	**PEG**
Phenol	5.2 -6.2	NPE
o-Cresol	5.6 – 6.6	5. 0 – 5.9
m-Cresol	5.2 – 6.2	5.2 – 6.0
p-Cresol	5.5 – 5.9	4.9 – 5.6
Aniline	5.1 – 5.8	NPE
o-Toluidine	5.1 – 6.4	NPE
m-Toluidine	4.7 – 5.7	NPE
p-Toluidine	5.1 -5.84	NPE

NPE – No PEG Effect. Range is defined by the width of the bell at 5.0% more substrate remaining than at the absolute minimum.

4.2 Optimum Laccase Concentration

Enzyme is a major cost to such a treatment regime so determining or predicting the minimum concentration needed in relation to pollutant concentration is desirable (Ibrahim et al., 2001; Al-Kassim et al., 1994). Experiments were performed within the optimum pH range varying both the substrate concentration within the range of 0.25 – 2.5

mM while increasing the laccase concentration until ≥ 95% removal of the initial substrate was achieved within the 3 h reaction period. It should be noted that the enzyme concentration and effectiveness can vary depending on retention times. When short contact times are required enzymes with high turnover rates that may also have high inactivation rates may be better suited than enzymes with lower turnover rates that may be more stable and less susceptible to inactivation and thus more suitable for reactors with long retention times. As with all optimization parameters, capital vs. operational costs must be balanced. Unlike the phenols, the aniline compounds were only run at one substrate concentration (1.0 mM). Initial substrate concentrations were kept low so that O_2 would not be limiting. The same set of experiments was run with the addition of a hydrophilic synthetic polymer, PEG, which has been demonstrated to reduce the required enzyme concentration with both laccase and peroxidases by prolonging enzyme activity (Ghosh et al., 2008; Saha 2006, Modaressi et al., 2005; Kulys et al., 2003; Caza et al., 1999; Wu et al., 1998). The mechanism by which PEG has the capacity to prolong enzyme activity is not known. The "PEG effect" has been proposed to follow the "sacrificial polymer theory" in which some of the insoluble products are attracted to it and thus preventing those polymers from adsorbing the free enzyme and settling it out of solution, or from the radicals themselves attacking the active site of the enzyme. (Nakamoto and Machida, 1992; Klibanov and Morris, 1980; Wu et al., 1998). Results can be found in **Fig 4.9-17** and are summarized in **Table 4.2.**

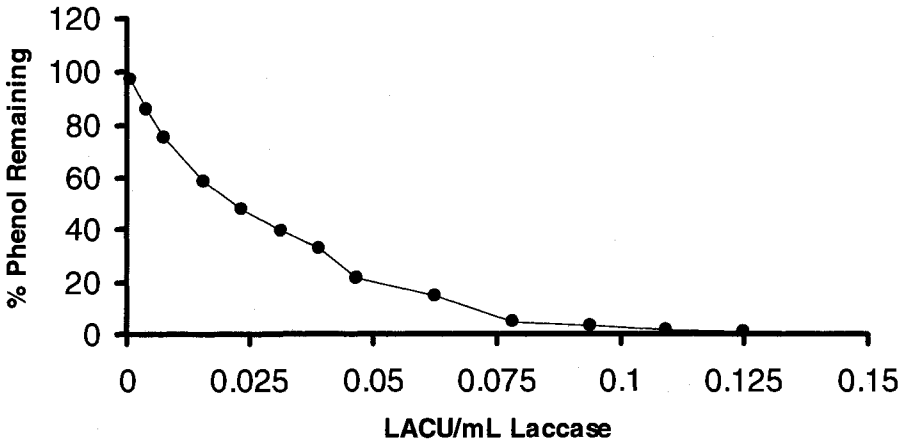
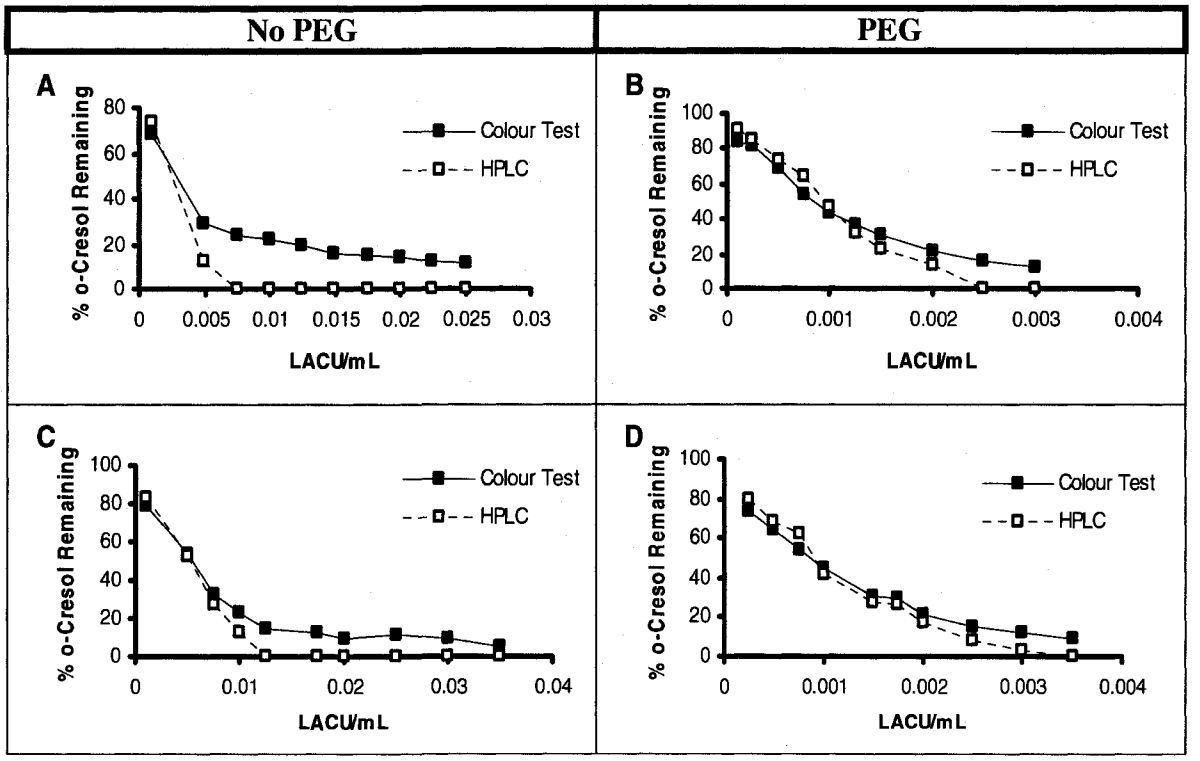


Figure 4.9: Laccase Concentration Optimization for 1.0 mM phenol. (3 h reaction; 40 mM phosphate buffer at 5.74).



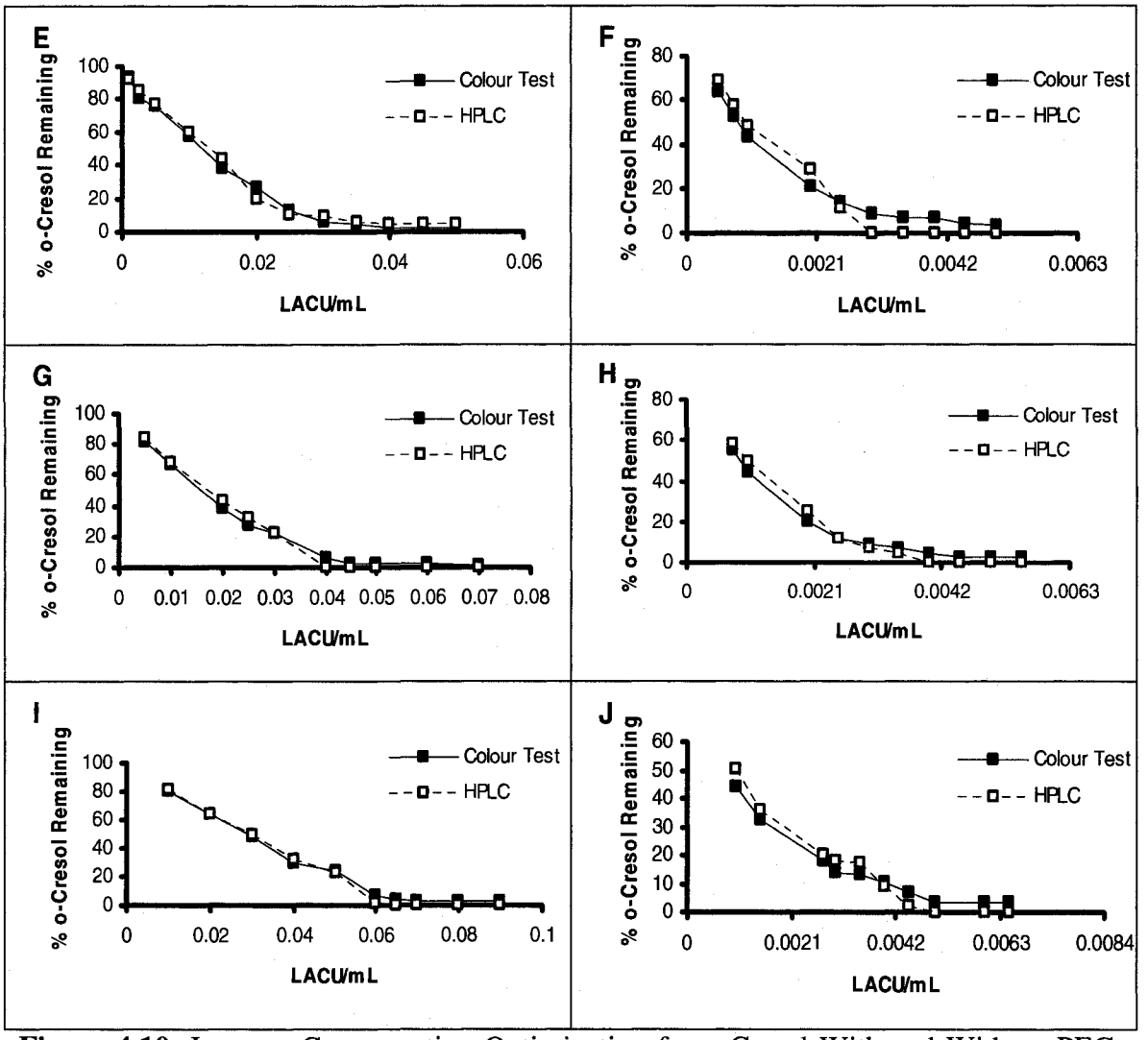


Figure 4.10: Laccase Concentration Optimization for o-Cresol With and Without PEG. (3 h reaction; 50 mM acetate buffer pH 5.65; initial o-Cresol concentration 0.25 mM for A,B, 0.5 mM for C,D, 1.0 mM E,F, 1.5 mM G,H, 2.0 mM I,J; PEG 100 mg/L added to reactions when required) All reactions without PEG illustrated in the left column and with in the right column.

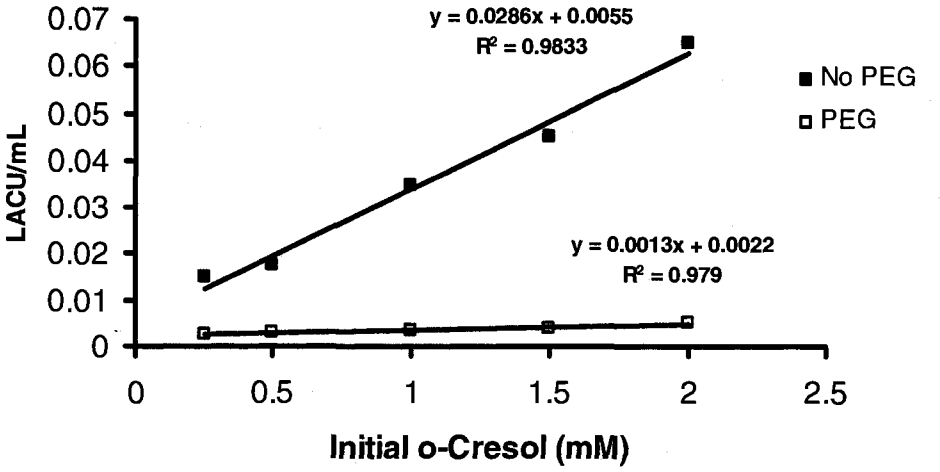


Figure 4.11: Laccase Concentration Optimization for o-Cresol With and Without PEG for Various Substrate Concentrations - analyzed by phenol colour test. Reaction conditions as stated in **Figure 4.10**.

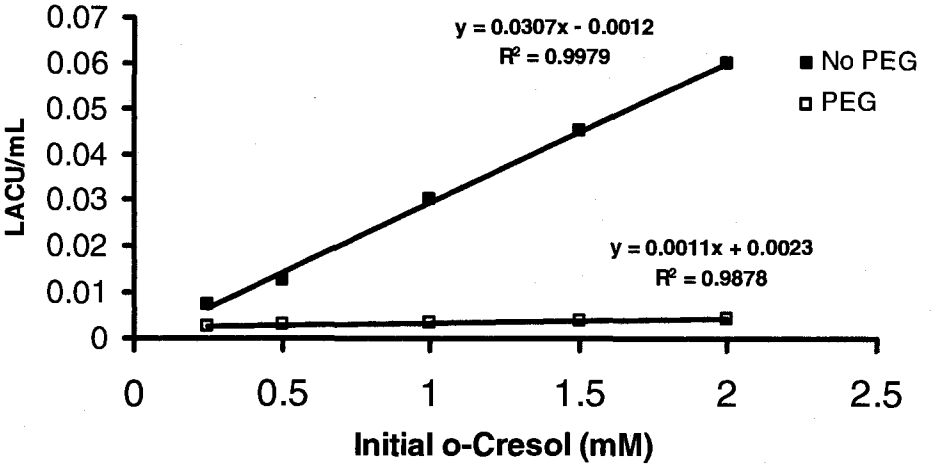
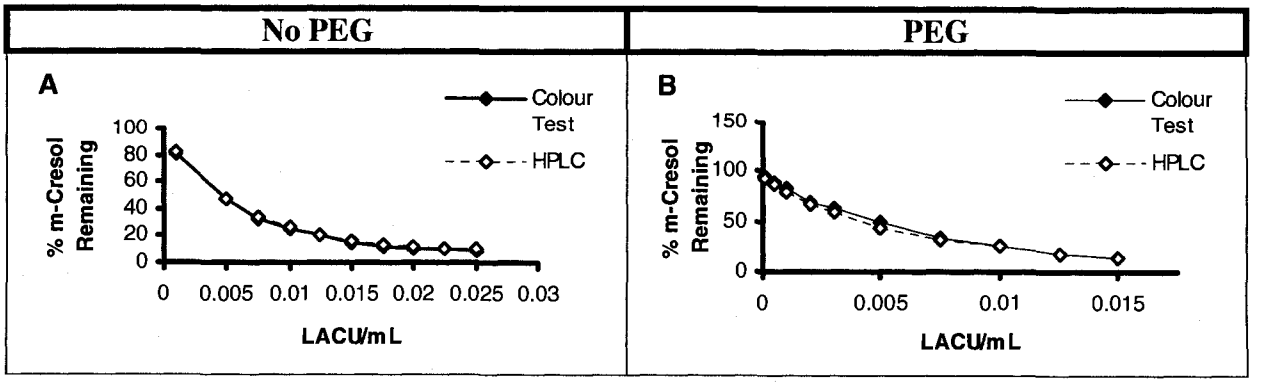


Figure 4.12: Laccase Concentration Optimization for o-Cresol With and Without PEG for Various Substrate Concentrations - analyzed by HPLC. Reaction conditions as stated in **Figure 4.10**.



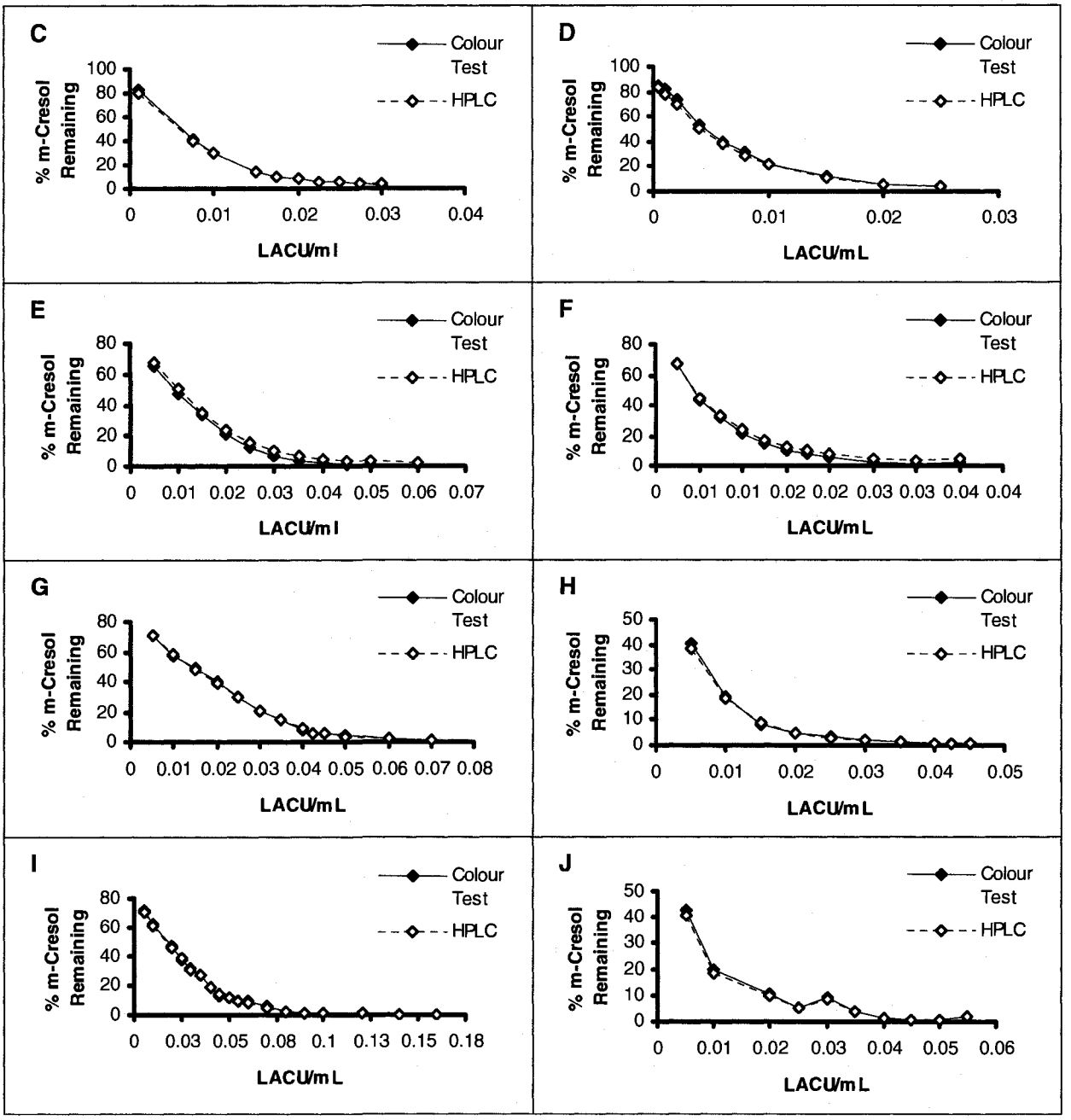


Figure 4.13: Laccase Concentration Optimization for m-Cresol With and Without PEG. (3 h reaction; 50 mM acetate buffers between 5.57- 5.65; initial m-Cresol concentration 0.25 mM for A,B, 0.5 mM for C,D, 1.0 mM E,F, 1.5 mM G,H, 2.0 mM I,J; PEG 100 mg/L added to reactions when required). All reactions without PEG illustrated in the left column and with in right column.

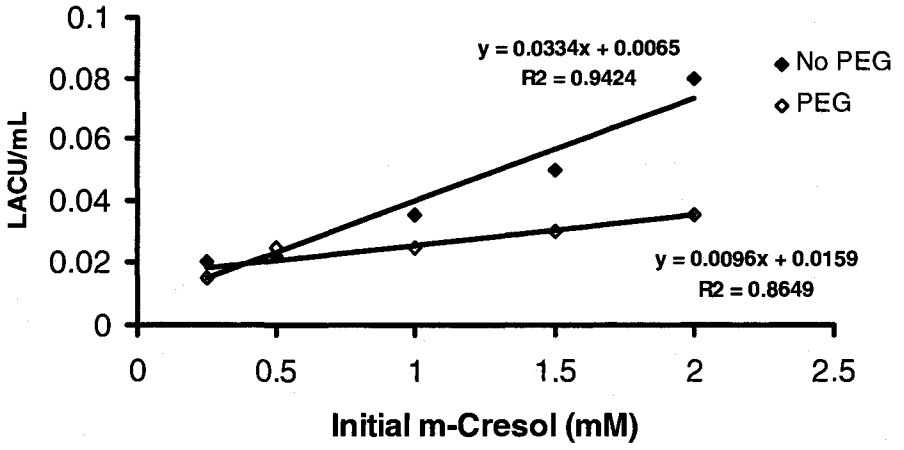


Figure 4.14: Laccase Concentration Optimization for m-Cresol With and Without PEG for Various Substrate Concentrations - analyzed by phenol colour test. Reaction conditions as stated in **Figure 4.13**.

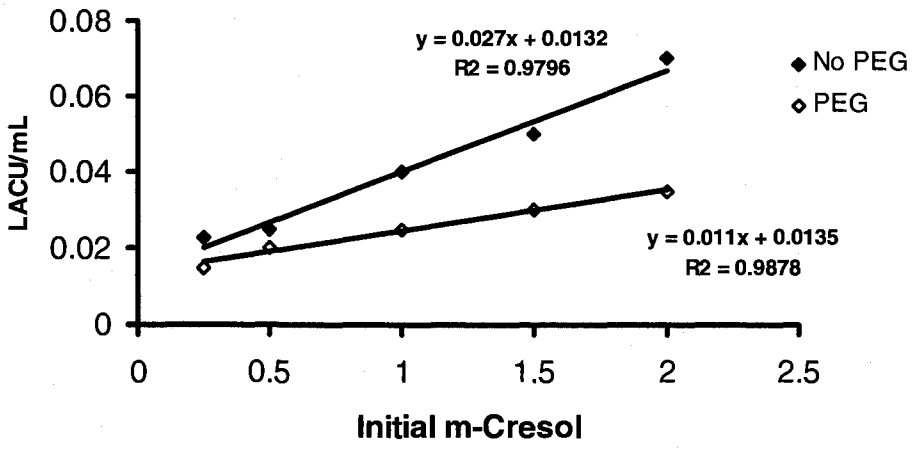


Figure 4.15: Laccase Concentration Optimization for m-Cresol With and Without PEG for Various Substrate Concentrations. Analyzed by HPLC. Reaction conditions as stated in **Figure 4.13.**

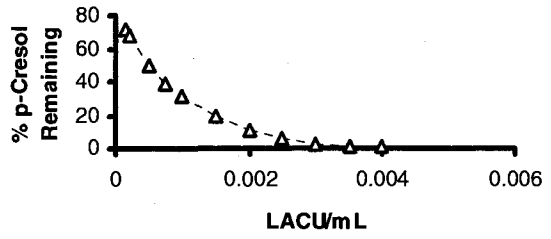
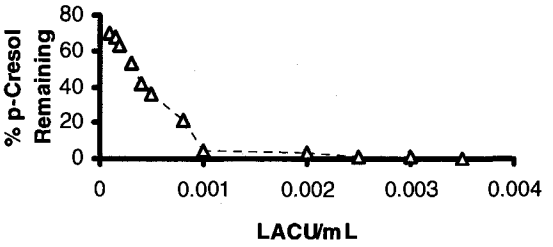
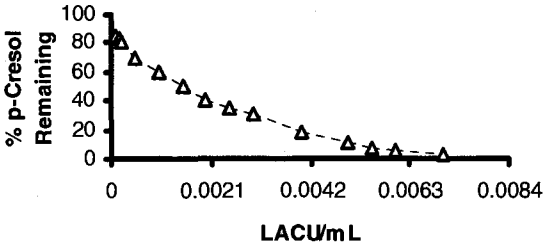
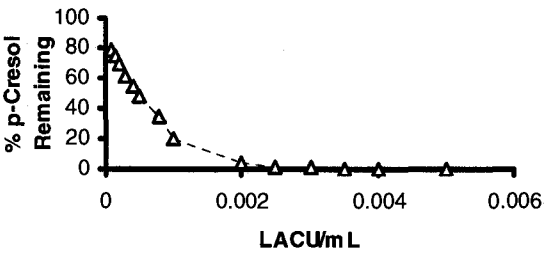
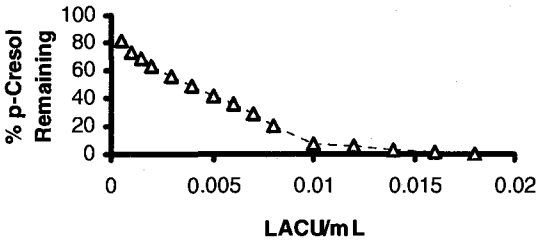
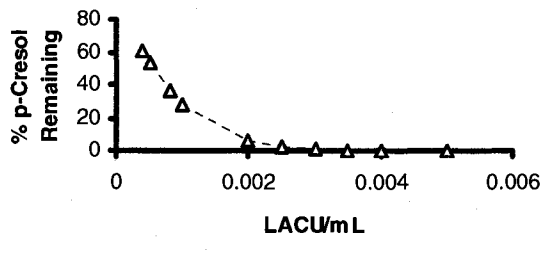
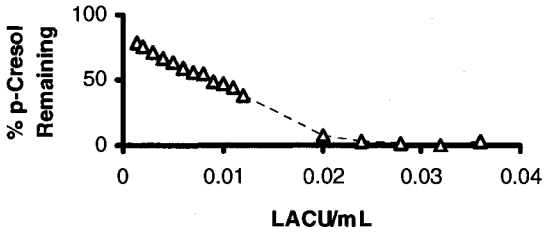
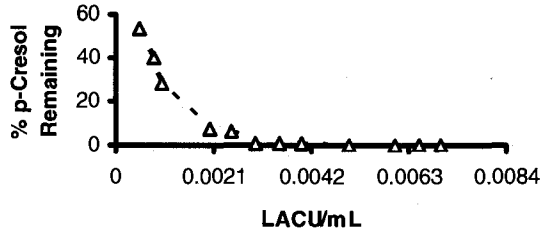
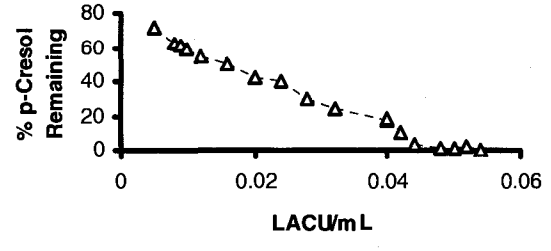
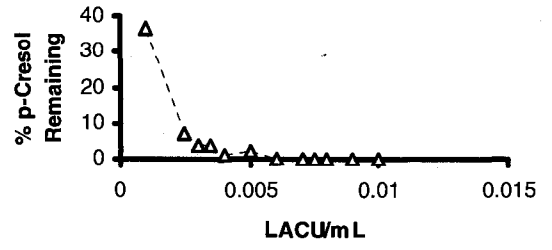
No PEG	PEG
A 	B 
C 	D 
E 	F 
G 	H 
I 	J 

Figure 4.16: Laccase Concentration Optimization for p-Cresol With and Without PEG. (3 h reaction, 50 mM acetate buffers between 5.62, initial p-Cresol concentration 0.25 mM for A,B, 0.5 mM for C,D, 1.0 mM E,F, 1.5 mM G,H, 2.0 mM I,J, PEG 100 mg/L added to reactions when required). All reactions without PEG illustrated in the left column and with in right column. **(Previous page)**

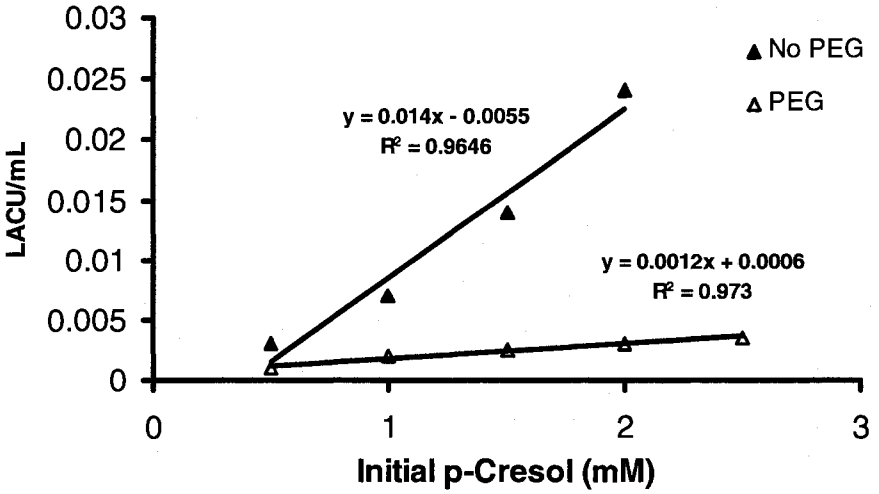


Figure 4.17: Laccase Concentration Optimization for p-Cresol With and Without PEG for Various Substrate Concentrations - analyzed by HPLC. Reaction conditions as stated in **Figure 4.16.**

Table 4.2: Minimum Nominal Laccase Concentration for ≥ 95% Conversion						
	o-Cresol (LACU/mL)		**m-Cresol (LACU/mL)**		**p-Cresol (LACU/mL)**	
Colour Test						
Initial Substrate (mM)	**No PEG**	**PEG**	**No PEG**	**PEG**	**No PEG**	**PEG**
0.25	0.015*	0.0025*	0.02*	0.015*	ND	ND
0.5	0.0175	0.003	0.0225	0.025	ND	ND
1.0	0.035	0.0035	0.035	0.025	ND	ND
1.5	0.045	0.004	0.05	0.03	ND	ND
2.0	0.065	0.005	0.08	0.035	ND	ND
HPLC						
0.25	0.0075	0.0025	0.0225	0.015	ND	ND
0.5	0.0125	0.003	0.025	0.02	0.003	0.001
1.0	0.03	0.0035	0.04	0.025	0.007	0.002
1.5	0.045	0.004	0.05	0.03	0.014	0.0025
2.0	0.060	0.0045	0.07	0.035	0.024	0.003
2.5	ND	ND	ND	ND	0.044	0.0035

ND- not done, * - only 90% removal achieved.

Table 4.3: PEG Effect						
Substrate	**Slope HPLC**		**Slope by Colour Test**		**Ratio No PEG : PEG**	
	No PEG	**PEG**	**No PEG**	**PEG**	**HPLC**	**C.T.**
o-Cresol	0.0307	0.0011	0.028	0.0013	27.91	22
m-Cresol	0.027	0.011	0.0334	0.0096	2.46	3.58
p-Cresol	0.014	0.0012	ND	ND	11.67	ND

ND- not done. Full equation for trend lines can be found in Figures **4.11, 4.12, 4.14, 4.15, 4.17.**

Phenol did not show increase in removal efficiency with the additive over the 3 h reaction as shown in **Fig. 4.73** over a range of 0-400 mg/L of PEG. The three cresol isomers did show a "PEG effect" that increased with increasing substrate concentration. As observed in **Fig. 4.11-12, 4.14 -15, 4.17**, linear relationships were found between minimum laccase concentration for ≥95% conversion with and without PEG. Colour test and HPLC results did vary slightly but could be attributed to the colorimetric assays' indiscriminate reactivity towards aromatic phenols with free p-position. Because of this it is expected that the phenol colour test gives elevated cresol concentrations and in the case of 0.25 mM phenols, the colour test reports only 90% removal while HPLC confirms ≥ 95 % conversion. The ratio of slopes (No PEG : PEG) shown in **Fig 4.11-12, 4.14-15, 4.17** are shown in **Table 4.3** and is an indication of the "PEG effect" for o,m,p-cresol. Within the range studied o-cresol had the largest 'PEG effect" of 27.9 while p-cresol and m-cresol had 11.7 and 2.46 slope ratios, respectively, determined by HPLC analysis. Similar results were observed with BPA in which a ratio of 5.2 was observed and for 1.0 mM 2,4 DMP in which a 2-fold reduction of laccase was allowed for ≥95% conversion (Modaressi et al., 2005; Ghosh et al., 2008). It should be noted that this PEG effect does not correspond to the actual decrease in enzyme in the batch reactor due to the y-intercepts in the equations of the line, however the ratio of the slopes is used for a more accurate comparison of PEG with the substrate. For example 1.0 mM o-cresol with PEG

requires 10 times less enzyme than without, not 27.9times less. The PEG requirement does increase linearly with increasing substrate concentration in the range studied. The addition of other water-soluble additives has also been demonstrated to retard enzyme inactivation of 1 and 2-napthol (Kulys et al., 2003). From the results we would anticipate that these trends would extend to higher concentrations until O_2 became a limiting factor to substrate conversion. The PEG effect has been reported up to ~200-fold decrease in enzyme for 10.0 mM phenol and 30-fold decrease for o-cresol conversion with HRP (Wu et al., 1998). All of the linear equations had positive intercepts except the o-cresol and p-cresol No PEG plots (HPLC analysis) (**Fig 4.12, 4.17**) which suggests some sort of error since substrate conversion requires some enzyme (**Table 4.3**). The negative intercepts imply that no enzyme for lower substrate concentrations would be required which is not plausible. We would expect a non-zero intercept in the PEG reactors because there is still significant residual enzyme activity remaing after the 3 h reaction period, as was observed with BPA (Kim and Nicell, 2006a). Also it should be noted that no "PEG effect" was observed with laccase SP 850 previously tested in our lab (Vermette 2000). The effects of replacing the substrate ring –OH group with a $-NH_2$ group were also investigated (**Fig 4. 18-21, Table 4.4**).

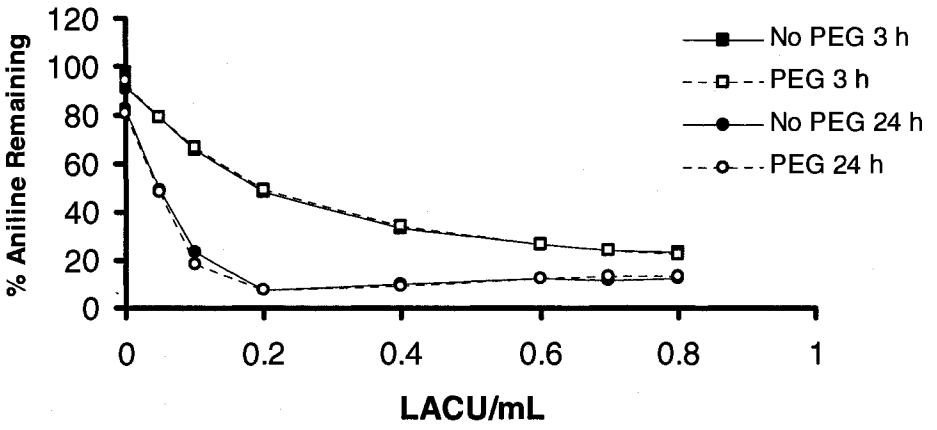


Figure 4.18: Laccase Concentration Optimization for 1.0 mM Aniline. (3 h reaction; 40 mM phosphate buffer, pH 5.82)

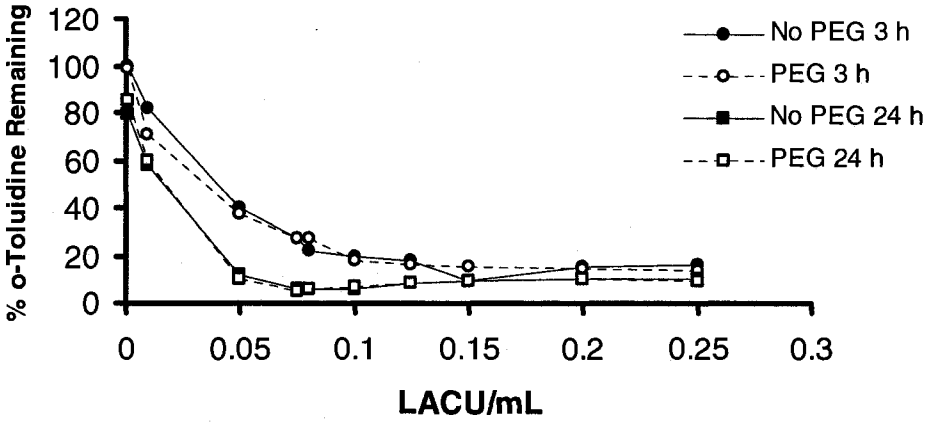


Figure 4.19: Laccase Concentration Optimization for 1.0 mM o-Toluidine. (3 h reaction,; 40 mM phosphate buffer, pH 5.82)

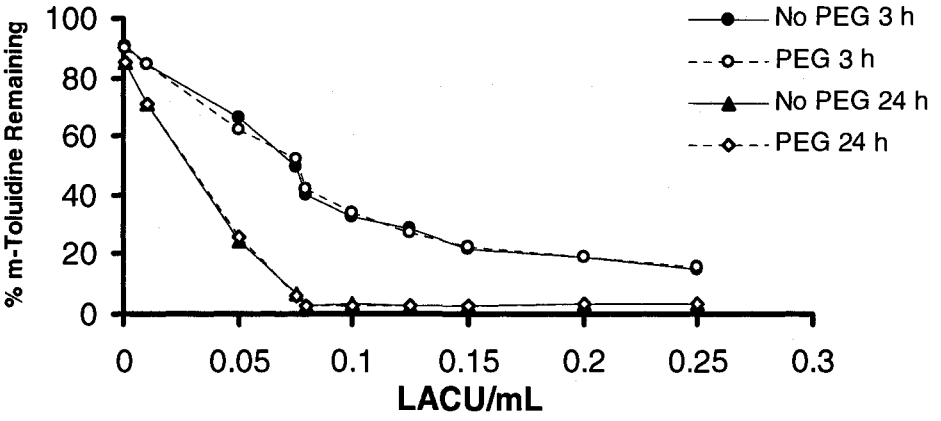


Figure 4.20: Laccase Concentration Optimization for 1.0 mM m-Toluidine. (3 h reaction; 40 mM phosphate buffer, pH 5.86)

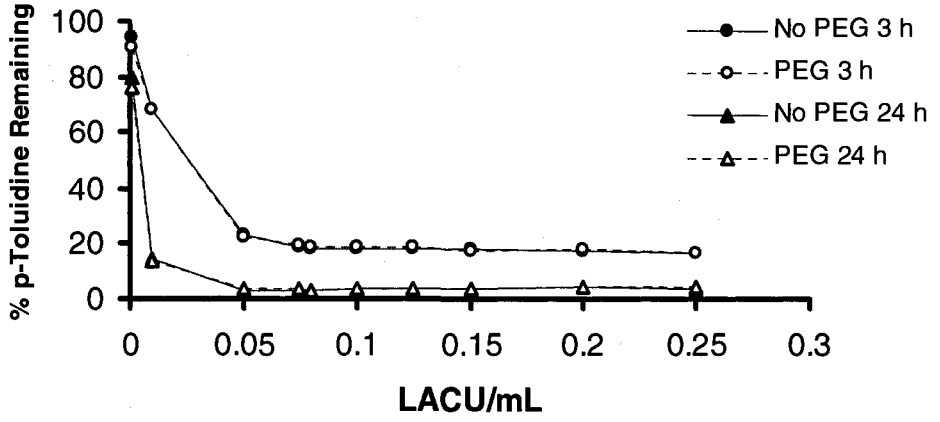


Figure 4.21: Laccase Concentration Optimization for 1.0 mM p-Toluidine. (3 h reaction; 40 mM phosphate buffer, pH 5.86)

It is quite apparent that the aniline compounds have a much higher laccase requirement than the phenolic compounds and even with 5-10 times more enzyme ≥ 95 % removal was not achieved within 3 h reaction periods (**Table 4.4**). In all cases removal seemed to level off when substrate was reduced to around 20% of the initial concentration. However if reaction time were extended to 24 hrs ≥ 95% removal for m- and p-toluidines is achieved proving that the enzyme has not been totally inactivated (more later). The

much higher concentrations of enzyme may have consumed the O_2 quicker thus limiting the reaction to the rate at which O_2 was diffusing into the water. Again it was demonstrated that there was no PEG effect with any of the anilines. It should be noted that currently no way of predicting substrate-enzyme combinations that will have a PEG effect have been identified based on the available data. At the time it seems to work with primarily phenolic compounds with SBP, HRP, and laccase SP504, with the exception of DPA-laccase SP504 which to our knowledge is the only secondary amine known to be tested (Ghosh et al., 2008; Modaressi et al., 2005; Wu et al., 1993).

Table 4.4: Optimum [E] for Removal of Various Substates (1.0 mM)

Substrate	Optimum [E] for 1.0 mM		
	No PEG (LACU/mL)	**PEG (LACU/mL)**	**24 h (LACU/mL**
Phenol	0.08	None	N.A.
o-Cresol	0.03	0.0035	N.A.
m-Cresol	0.04	0.025	N.A
p-Cresol	0.007	0.002	N.A
Aniline	0.8‡	None	0.2†
o-Toluidine	0.15‡	None	0.075*
m-Toluidine	0.25‡	None	0.075
p-Toluidine	0.075‡	None	0.05

*Removal to 90 %, † Removal to 85%, ‡ Removal to 80 %, N.A. Not Applicable)

When looking at the effect of positioning of the functional groups (-OH or $-NH_2$) it is observed that the parent compounds phenol or aniline require the most enzyme followed by m-, o-, p-substitution patterns. Numerous research groups have correlated higher activity laccases with higher redox potentials which may explain why fungal laccases (E° 0.48 – 0.8V) vs. plant laccases (E° ~ 0.430 – 500 mV) are more reactive (Xu et al., 1996). Thus it may be possible to predict the effectiveness of multicopper oxidases and efficiency of substrate conversion to a certain degree. This increase in redox potential is thought to be related to the coordination sphere of the T1 Cu site as compared to other

multicopper oxidases with lower redox potentials (Xu et al., 1999; Xu et al., 1997; Tadesse et al., 2008; Xu 1996). Plastocyanin (Pc) (blue copper oxidase) and zucchini ascorbate oxidase (zAO) (multi-copper oxidase) both have significantly lower E° in the range of 0.3-0.4 V with their T1 Cu's bound to two His and a Cys with a short C-S bond and a Met with a long C-S bond forming a distorted tetrahedral coordination geometry (Xu et al., 1999). While fungal laccases are usually found to have Leu or Phe replacing the axial Met that is not expected to co-ordinate the T1 Cu thus leaving a tri-coordinated T1 Cu site (Xu et al., 1999). Stronger axial ligands would stabilize the Cu^{+2} (lower E°) whereas weaker axial ligands would have less affinity for the Cu^{+2} thus increasing the E° (Xu et al., 1998). Mutations in *Trametes villosa* by replacing Phe463 with Met showed a decrease in redox potential by 0.1 V as well as increased K_M and k_{cat} values in oxidizing phenols attributed to the perturbation of T1 Cu geometry due to ligation (Xu et al., 1999). Knowing this may also lead to ways of tuning these multicopper oxidases to encompass an even larger variety of substrates or possibly further reduce the quantity of enzyme needed, however, engineering of the enzyme would add to the cost. Xu (1996) displayed the relationship between the log (k_{cat}/K_M) with $\Delta E°$ = [(E° pH 5 of laccase T1Cu) – (E° substrate)] for both substituted phenols an anilines and has been confirmed by Tadesse et al., 2008. Both studies show that the specifity constant (k_{cat}/K_M) decreased with increasing redox potentials of substrate. It was also shown that within a substrate series K_M increased with less reducing substrates suggesting a lower affinity (Tadesse et al., 2008). In agreement with this study laccases seem to show a higher affinity for phenols than anilines when K_M values are compared, even when redox potentials are very similar (p-cresol and p-toluidine 0.79) (Tadasse et al., 2008; Munoz et al., 1997). Benzenethiols

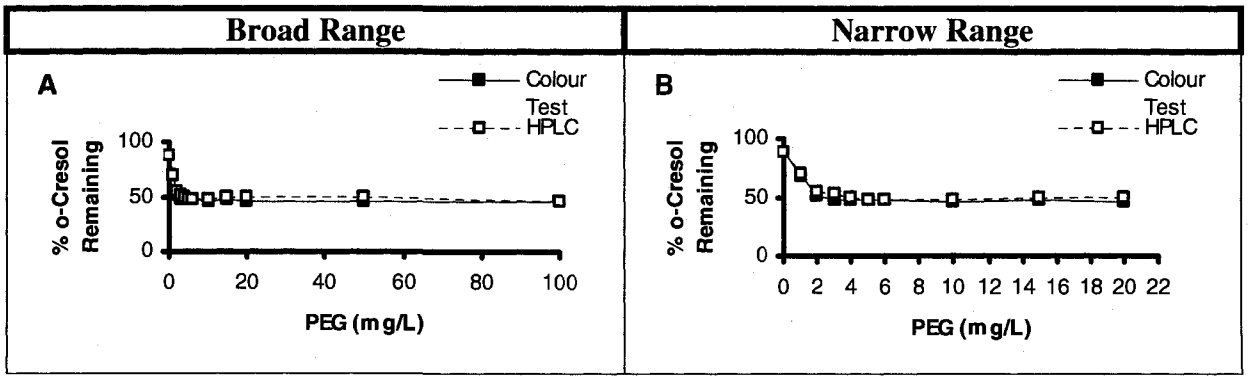
were not examined in this study but it should be noted that they have not been observed to follow the same trend as the phenols and anilines and it is thought that the thiol group may have a unique interaction with the T1 Cu (sulfur-containing groups often have high affinity for Cu ions) (Xu et al., 1996).

Other considerations when selecting target substrates for enzymatic treatment are the addition of electron-withdrawing and -donating groups, the size and positioning of substituents relative to the functional group (-OH, $-NH_2$, -SH). Electron-withdrawing substituents diminish phenol conversion and usually have increased K_M and decreased k_{cat} values, speculated to be caused by the reduction in electron density around the phenoxyl group (harder to oxidize) (Xu 1996). These substrates tend to have higher redox potentials as well as being less basic, shown by smaller pK_a values. Another study comparing 4 fungal laccases with the conversion of methoxy phenols (*Coriolus hirsutus*, *Coriolosmus fulvocinerea*, *Coriolus zonatus*, *Cerrena maxima*) speculated that both inductive and mesomeric effects contribute to the kinetic phenomenon (Smirnov et al., 2001). This was supported by the comparison of guaiacol, ferulic acid, and sinapinic acid in which it was speculated that CH=CH-COOH in the para position contributes to a negative mesomeric effect on ferulic and sinapinic acid but the conjugated system would aid in the delocalization of electron density stabilizing radical formation, increasing catalytic effectiveness (Smirnov et al., 2001). Also the addition of a methoxy group to ferulic acid to make sinapininc acid would have a positive mesomeric effect also contributing to increased catalytic efficiency (Smirnov et al., 2001). The methoxy groups are also electron donating groups and may have nothing to do with mesomeric effects but ultimately just be inductive. By substituting bulky t-butyl groups onto phenols in

various positions of the ring it was shown that p-substituted phenols showed no effect whereas the 3,5-di(Bu^t)phenol and 3,5-di(Bu^t)aniline "obstructed approach and satisfactory π-stacking interactions" with His458 (Tadesse et al., 2008). o-Substituent groups can also be problematic, however, in the present study, electronic contributions of the smaller –OH and $–NH_2$ groups would play a larger role on activity than steric effects.

4.3. PEG Optimization

PEG is a hydrophilic synthetic polymer and, like all additives in wastewater treatment, must be accounted for. Thus the optimum PEG concentration, defined as the minimum concentration necessary for maximum effect after which point further addition of PEG would not significantly improve substrate conversion was determined for the three cresol isomers over a concentration range of 0.5 – 5.0 mM. All reactions were run under stringent conditions with respect to enzyme concentration, under optimum and increasing PEG concentrations. All samples were run in triplicate, except p-cresol which was only run in a duplicate set of batch reactors. Within the substrate range studied a linear trend was found in relation to PEG:substrate ratio **Fig 4.23, 4.25,4.27**.



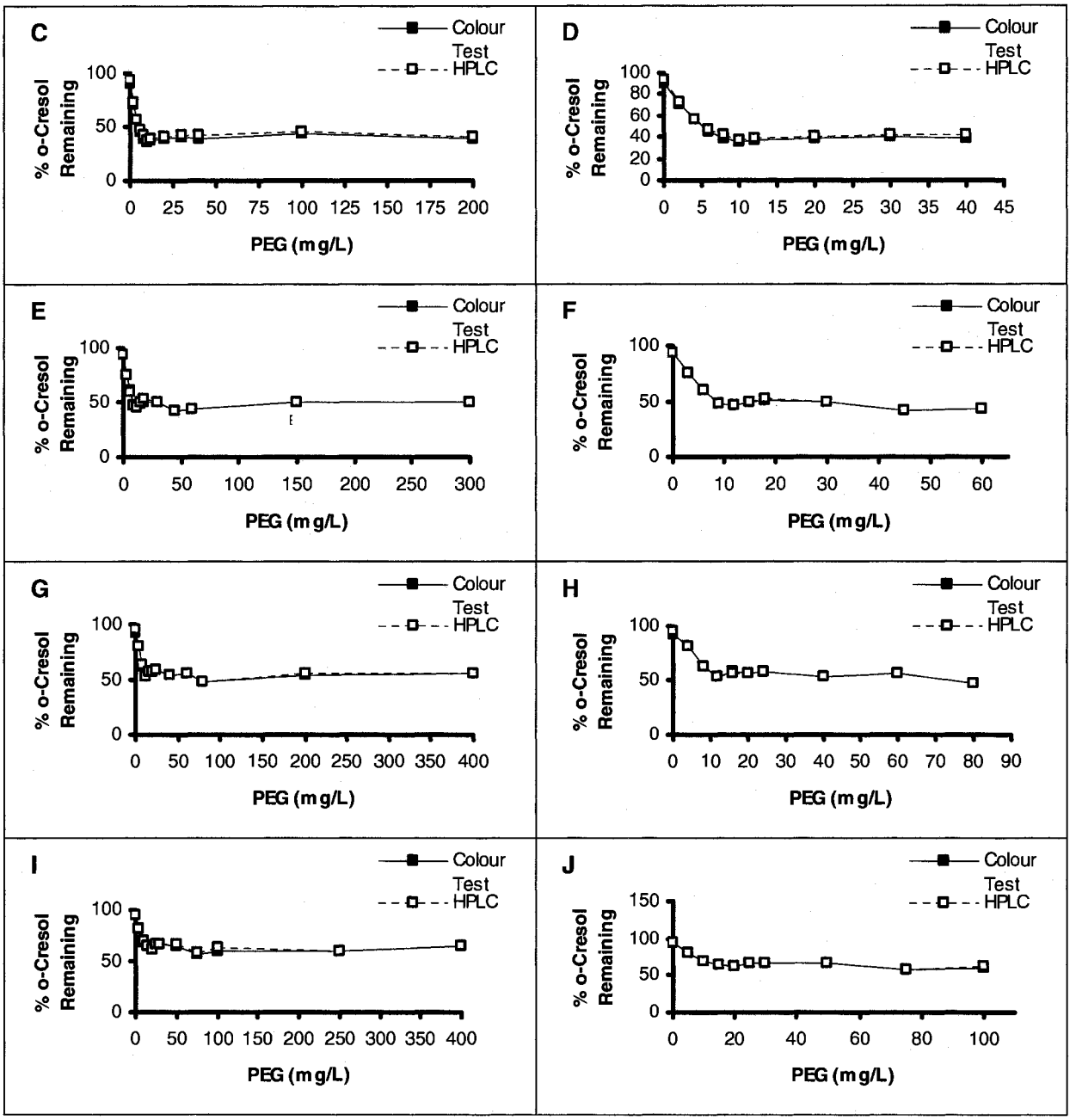


Figure 4.22: PEG Optimization for Various o-Cresol Concentrations. (3 h reaction; 40 mM acetate buffer, pH 5.64; 1.0 mM o-cresol and 0.0015 LACU/mL for A,B, 2.0 mM o-cresol and 0.002 LACU/mL for C,D, 3.0 mM o-cresol and 0.0025 E,F, 4.0 mM o-cresol and 0.003 LACU/mL G,H, 5.0 mM o-cresol and 0.004 LACU/mL I,J)

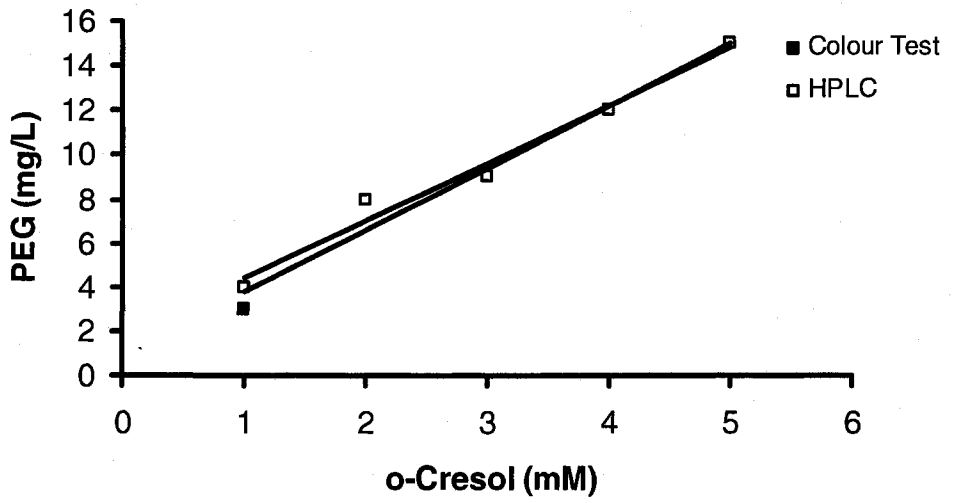
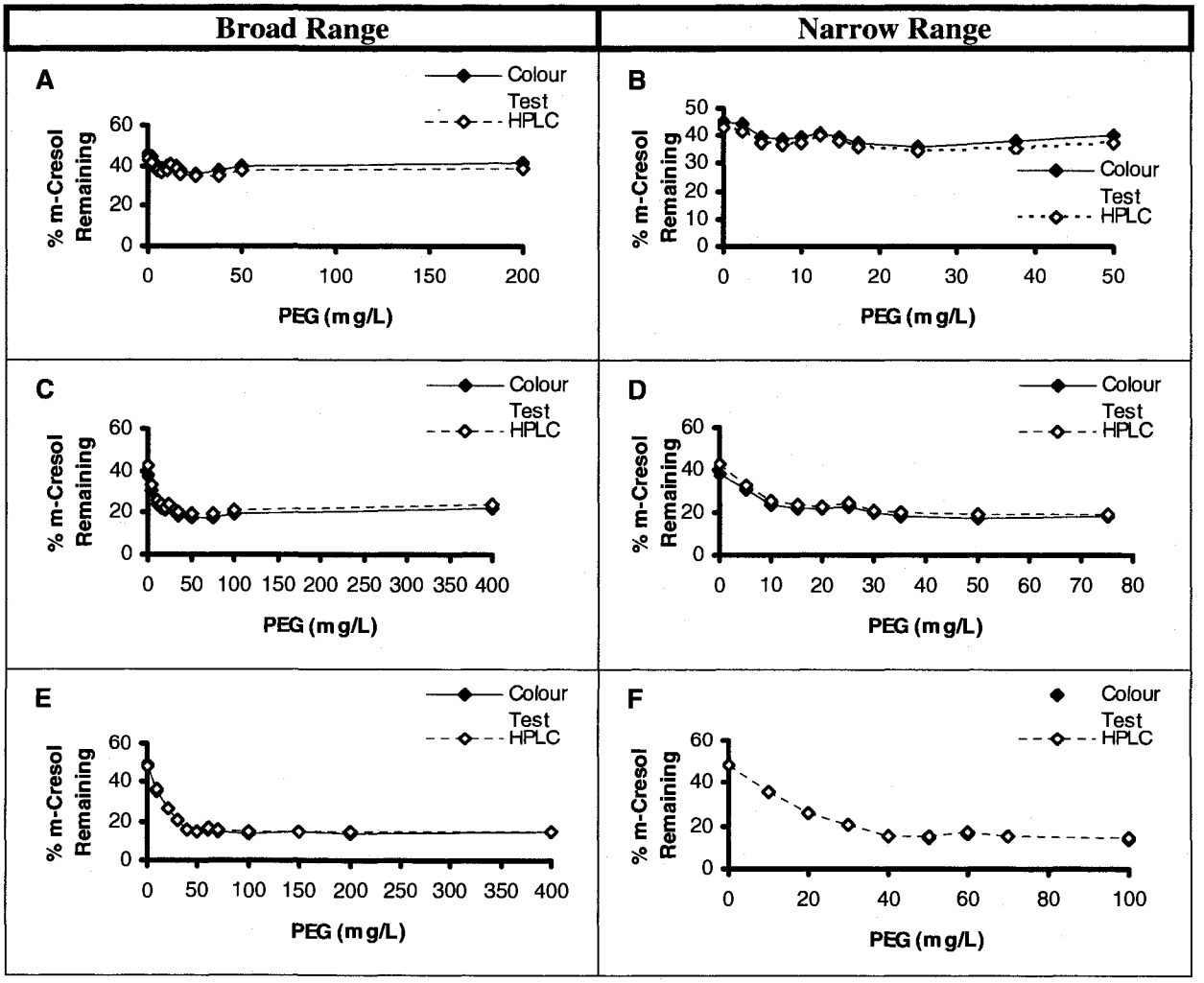


Figure 4.23: Minimum PEG to o-Cresol Ratio. Results were determined from **Figure 4.22**. Colour Test: $y = 2.8x + 1$, $R^2 = 0.9655$. HPLC: $y = 2.6x + 1.8$, $R^2 = 0.9769$.



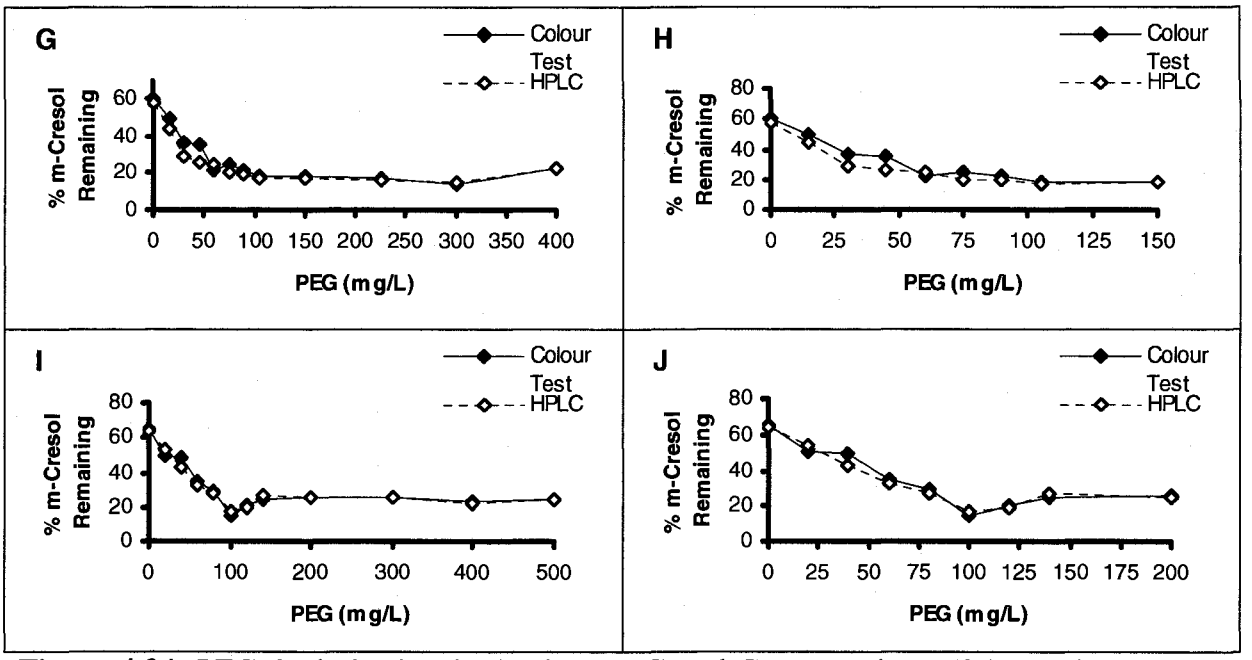


Figure 4.24: PEG Optimization for Various m-Cresol Concentrations. (3 h reaction; 40 mM acetate buffer, pH 5.64; 0.5 mM m-cresol and 0.0075 LACU/mL for A,B, 1.0 mM m-cresol and 0.0125 LACU/ mL for C,D, 2.0 mM m-cresol and 0.0175 E,F, 3.0 mM m-cresol and 0.02 LACU/mL G,H, 4.0 mM m-cresol and 0.025 LACU/mL I,J)

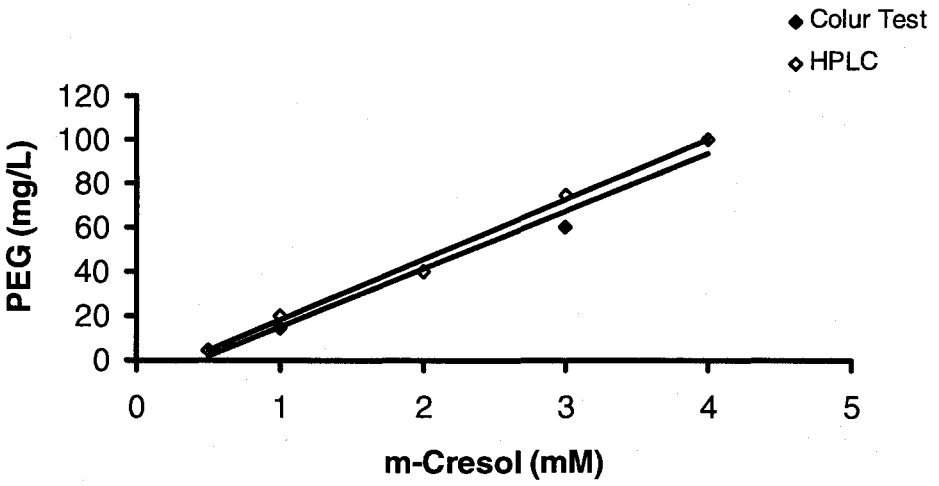


Figure 4.25: Minimum PEG to m-Cresol Ratio. Results were determined from **Figure 4.24**. Colour Test: $y = 26.28x - 11.189$, $R^2 = 0.9815$. HPLC: $y = 27.256x - 9.2378$, $R^2 = 0.9938$.

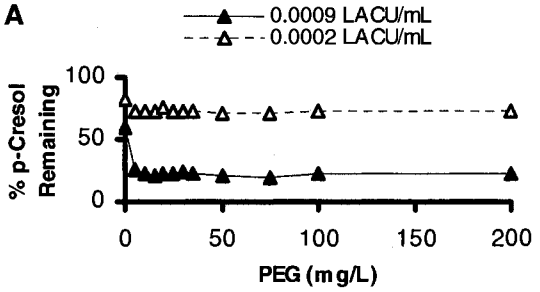
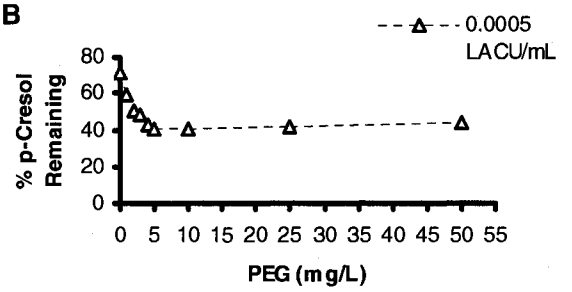
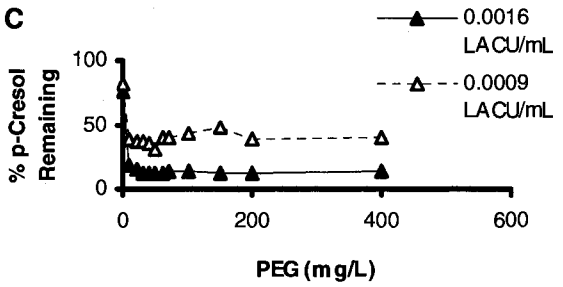
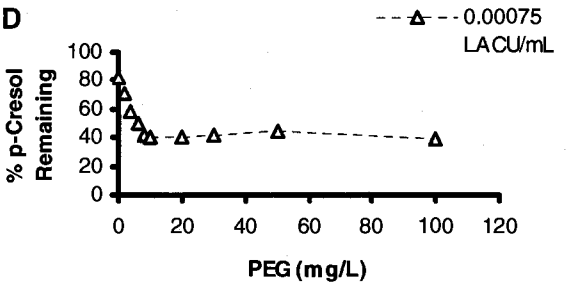
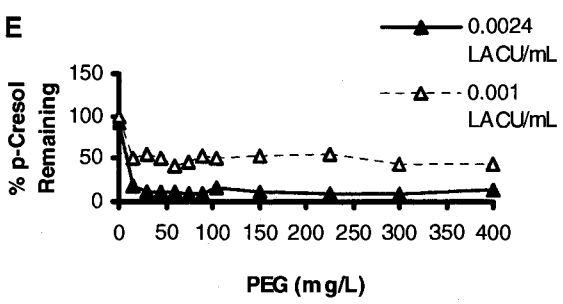
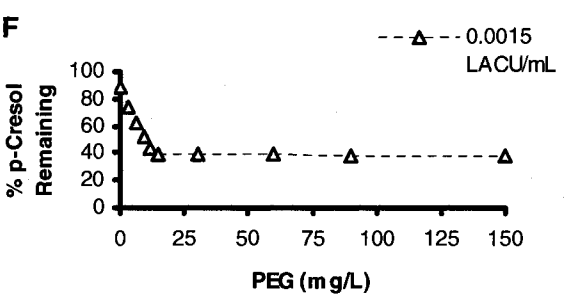
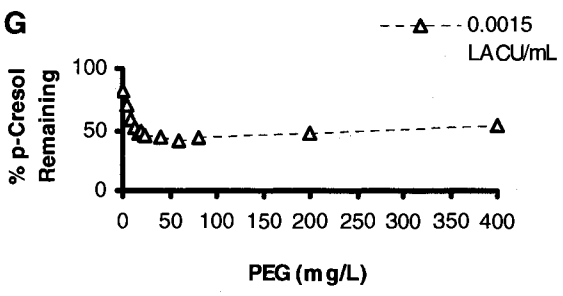
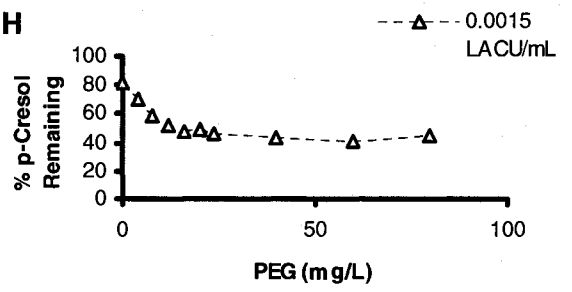
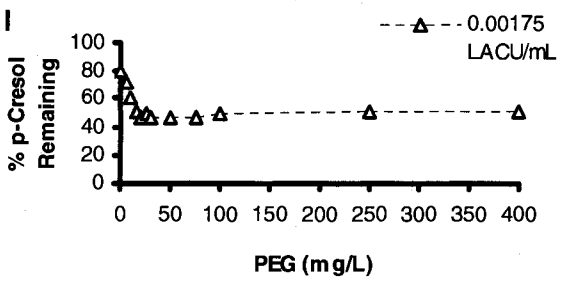
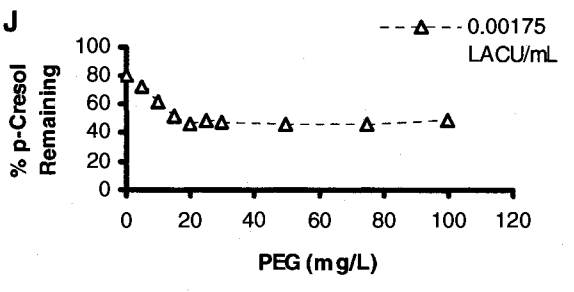
Broad Range	Narrow Range
A 	B 
C 	D 
E 	F 
G 	H 
I 	J 

Figure 4.26: PEG Optimization for Various p-Cresol Concentrations. (3 h reaction; 40 mM acetate buffer, pH 5.68; 1.0 mM p-cresol for A,B, 2.0 mM p-cresol for C,D, 3.0 mM p-cresol for E,F, 4.0 mM p-cresol for G,H, 5.0 mM p-cresol for I,J) **(Previous page)**

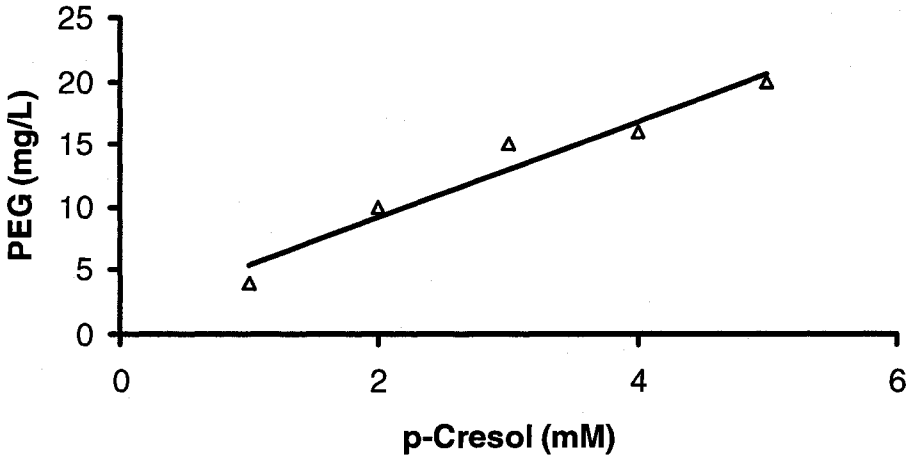


Figure 4.27: Minimum PEG to p-Cresol Ratio. Results were determined from **Figure 4.26**. HPLC: $y = 3.8x + 1.6$, $R^2 = 0.9500$.

Table 4.5: Minimum PEG Concentration for Optimum "PEG Effect"					
Initial Substrate (mM)	**o-Cresol -PEG (mg/L)**		**m-Cresol-PEG (mg/L)**		**p-Cresol –PEG (mg/L)**
	HPLC	**C.T.**	**HPLC**	**C.T.**	**HPLC**
0.5	ND	ND	5	5	ND
1.0	4	3	20	15	4
2.0	8	8	40	40	10
3.0	9	9	75	60	15
4.0	12	12	100	100	16
5.0	15	15	ND	ND	20

C.T. - phenol colour test results, ND- not done, Values reported as an average.

Table 4.6: Trend lines to Predict PEG:Substrate Ratios			
Analytical Method	**o-Cresol**	**m-Cresol**	**p-Cresol**
HPLC	$y = 2.6x + 1.8$ $R^2 = 0.9769$	$y = 27.26x - 9.24$ R^2 $= 0.9938$	$y = 3.8x + 1.6$ $R^2 = 0.9500$
Colour Test	$y = 2.8x + 1$ $R^2 = 0.9655$	$y = 26.28x - 11.19$ $R^2 = 0.9815$	ND

ND- not done, Values reported as an average. y = PEG (mg/L), x = cresol (mM)

By plotting the initial substrate concentration versus the optimum PEG concentration linear plots are obtained with R^2 values ranging from 0.9800-0.9938 and both colour and

HPLC analysis in close agreement (**Table 4.5-6**). The slopes of the lines represent the approximate concentration of PEG mg/L per mM cresol. m-Cresol had a much higher PEG demand than o- and p-cresols. It is noteworthy that these concentrations, **Table 4.7** (page 106) are much less than the reported values for HRP and SBP (**Table 1.5a and 1.5b**). The negative intercept for m-cresol is a bit worrisome since this would suggest that no PEG is required when substrate concentration is low. However if PEG does operate as the "sacrificial polymer theory" suggests this could be explained by the fact that m-cresol is showed less inactivation under the optimum reaction conditions (**Figure 4.45**) or perhaps the trend does not apply to the lower m-cresol concentrations. The PEG effect is a smaller factor with decreasing substrate concentration, mainly because inactivation is considered to increase proportionally with increasing substrate concentration (Wu et al., 1998). Using the same laccase it was found that optimum PEG concentrations of 146 and 1.0 mg/L were determined for 1.0 mM BPA and 2,4 DMP, respectively (Modaressi et al., 2005; Ghosh et al., 2008). 50 mg/L of PEG_{40000} was shown to extend the life of laccases during conversion of 1- and 2-napthol but was not optimized, only the single concentration run (Kulys et al., 2003). Since the exact mechanism as to how PEG protects the enzyme is not known it is hard to explain why some substrates require so much less PEG than others, or why m-cresol would require almost ten times more PEG than the o- and p-isomers. In the original study by Nakamoto and Machida (1992) it was also observed that increasing substrate concentrations required increasing concentrations of gelatin. The increase in additive demand supports the "sacrificial polymer theory" because increasing initial substrate concentration in turn would increase the amount of reactive intermediates and or polymeric products thought to

be the source of inactivation. The linear trends support a stoichiometric relationship between polymeric product and PEG.

To again confirm that PEG had no effect on the aniline compounds a similar set of experiments were run and conversion was monitored at 3 and 24 h. The reasoning behind this was based on the observation that precipitate took much more time to build up in the reactors and thus if the "sacrificial polymer theory" is true we would not expect to see a decrease in conversion as quickly since there would be nothing in solution to adsorb the free enzyme. If inactivation was slow, a longer reaction time would help accentuate any effect PEG had. All inactivation would have to be by other mechanisms such as radical attack on the active site.

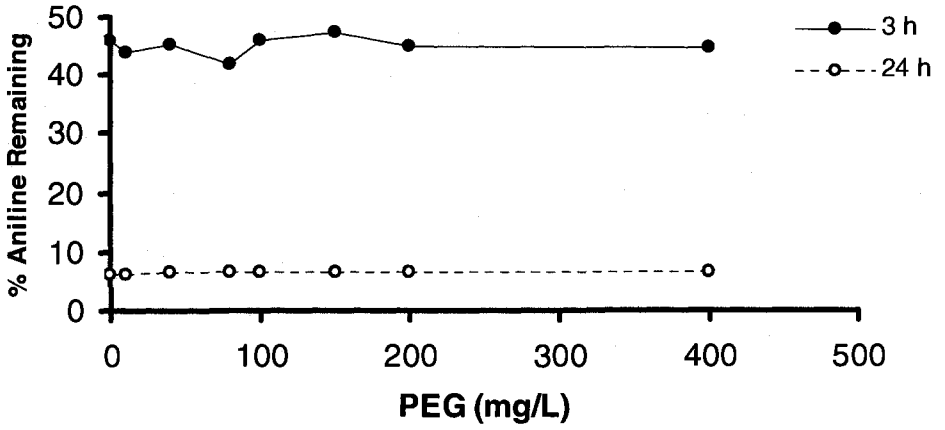


Figure 4.27: PEG Optimization for Aniline. (1.0 mM Aniline; 3 h reaction; 40 mM phosphate buffer, pH 5.88; 0.200 LACU/mL)

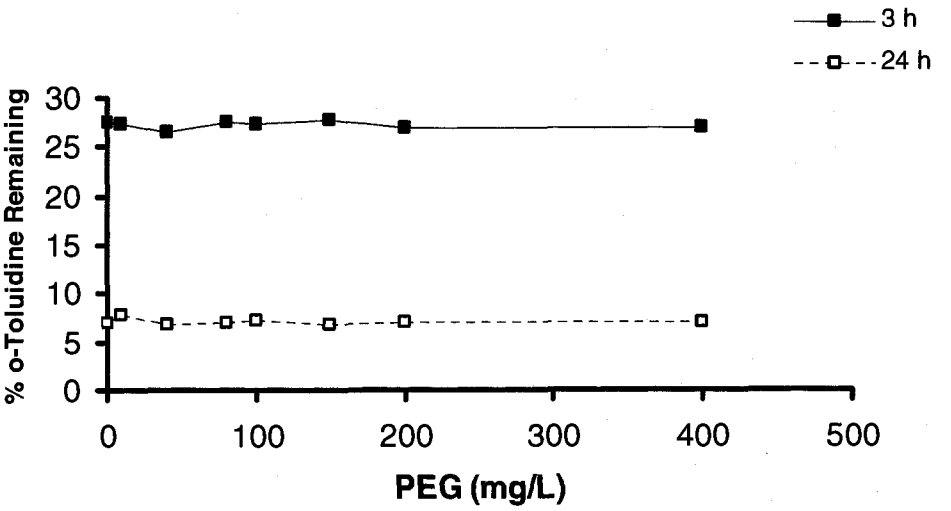


Figure 4.28: PEG Optimization for o-Toluidine. (1.0 mM o-Toluidine; 3 h reaction; 40 mM phosphate buffer, pH 5.88; 0.075 LACU/mL)

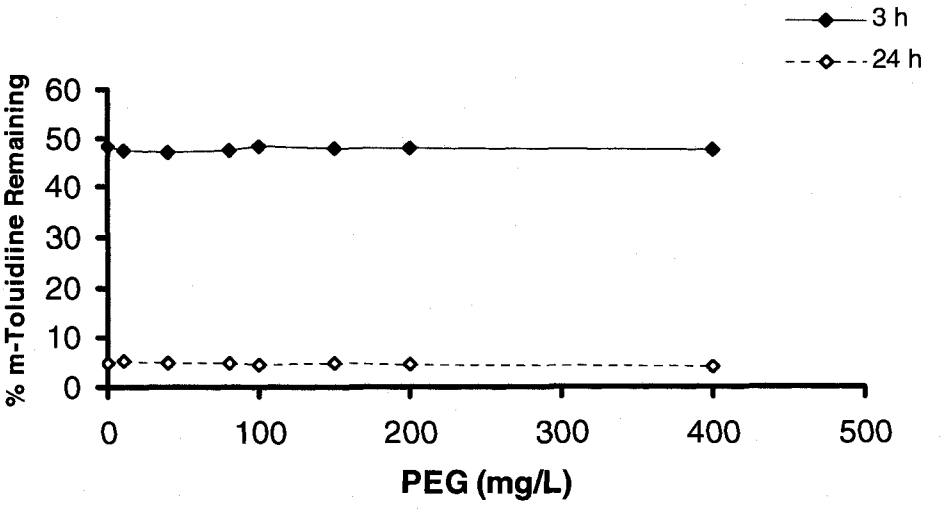


Figure 4.29: PEG Optimization for m-Toluidine. (1.0 mM m-Toluidine; 3 h reaction; 40 mM phosphate buffer, pH 5.88; 0.08 LACU/mL)

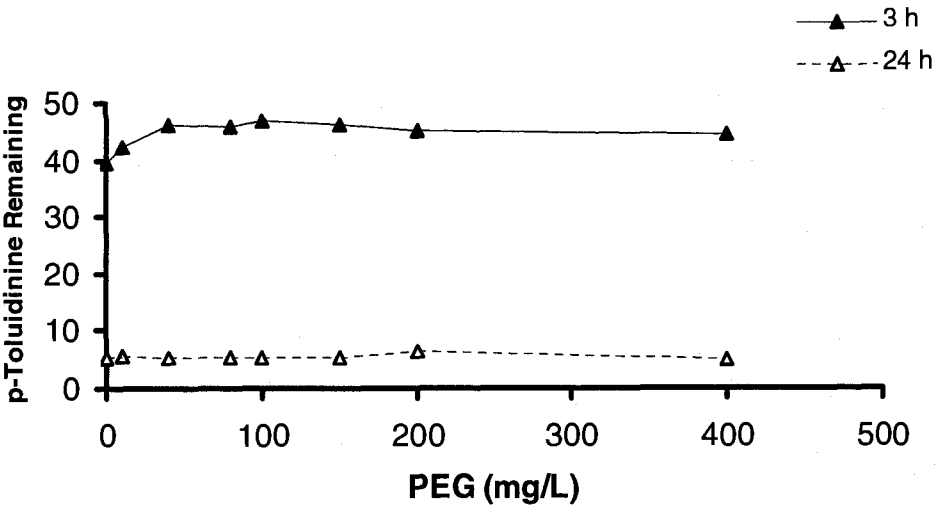


Figure 4.30: PEG Optimization for p-Toluidine. (1.0 mM p-Toluidine; 3 h reaction; 40 mM phosphate buffer, pH 5.88; 0.025 LACU/mL)

Even after 24 h the reactors with PEG showed no difference in conversion from the control, even a negative effect with aniline (**Fig. 4.30**). It appeared as if there was no solid particulate in the reactors however, after microfiltration the membranes we noticeable coloured. The filtrate was still very coloured despite this.

4.4 PEG Molecular Weight

Nakamoto and Machida (1992) first proposed using additives to suppress HRP inactivation with the thought that polymerized phenols, or their intermediates would have the same type of interaction(s) with other proteins. Thus these other proteins should be able to attract some of the harmful intermediates, or polymerized phenols thought to be inactivating HRP away from the enzyme, prolonging its lifetime, increasing turnover and therefore reducing cost of the enzyme treatment (sacrificial polymer theory). Studies started with the addition of gelatin (mixture of proteins) that was optimized at 4 g/L for 10 g/L of phenol. Later, hydrophilic synthetic polymers were investigated and one of particular interest was PEG. It was interesting that PEG with an average MW below 600

demonstrated no improvement on turnover. PEG MW was optimized at 1000 (Nakamoto and Machida, 1992). Since then, similar results have been observed as well as varying catalytic efficiencies with different molecular weights of PEG (Wu et al., 1998; Saha 2006; Kinsely and Nicell, 2000). SBP conversion seemed to increase almost indefinitely with the increasing MW of PEG (Kinsely and Nicell, 2000). If larger PEG chains are more efficient in protecting the enzyme and lower concentrations of PEG can be used the overall cost of enzymatic treatment can be reduced. Thus PEG MW and quantity may not be independent of each other. Smaller chain PEG may not form complexes as efficiently and therfore larger amounts may not have effect. The fact that the same quantities of PEG's with varying molecular weights have different effects also implies that they are independent from one another. Reactors were made with 1 mM o-,m-, or p-cresol, and run at optimum pH, under stringent conditions with respect to laccase concentration and increasing PEG concentrations from 0-100 mg/L. o- and m–Cresols were run in triplicate sets of batch reactors and analyzed for residual substrate by colorimetric assay at 3 h, as well as 14, 28 h for o-cresol. p-Cresol was run in duplicate and analyzed at 3 and 24 h by HPLC. Results can be found in **Fig. 4.31-35** and summarized in **Table 4.7**

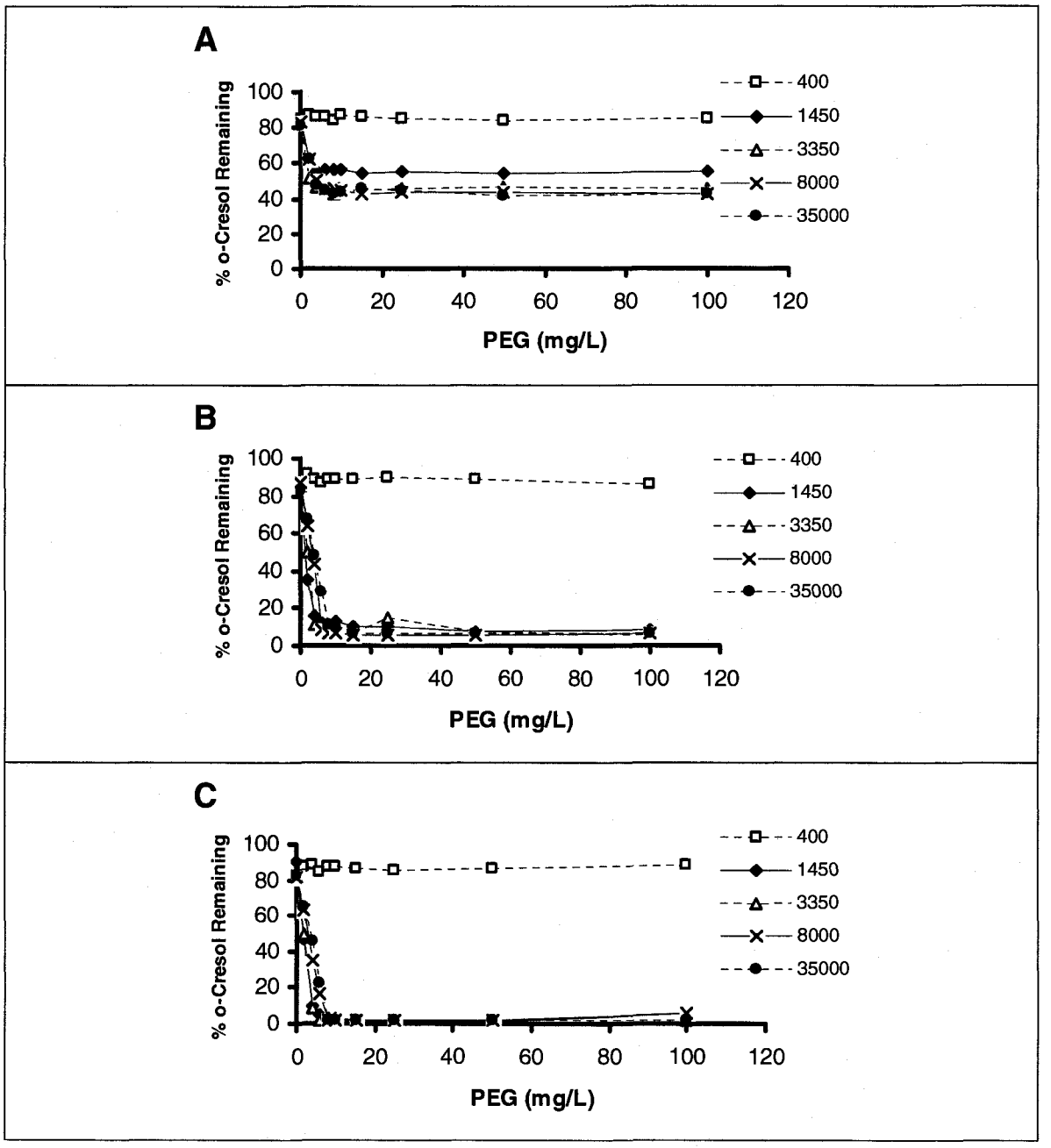


Figure 4.31: Molecular Weight Optimization for PEG with o-Cresol Broad Range. (1.0 mM o-cresol; 3 h reaction (A), 14 h (B), 28 h (C); 40 mM acetate buffer, pH 5.68; 0.0015 LACU/mL; only analyzed by phenol colour test)

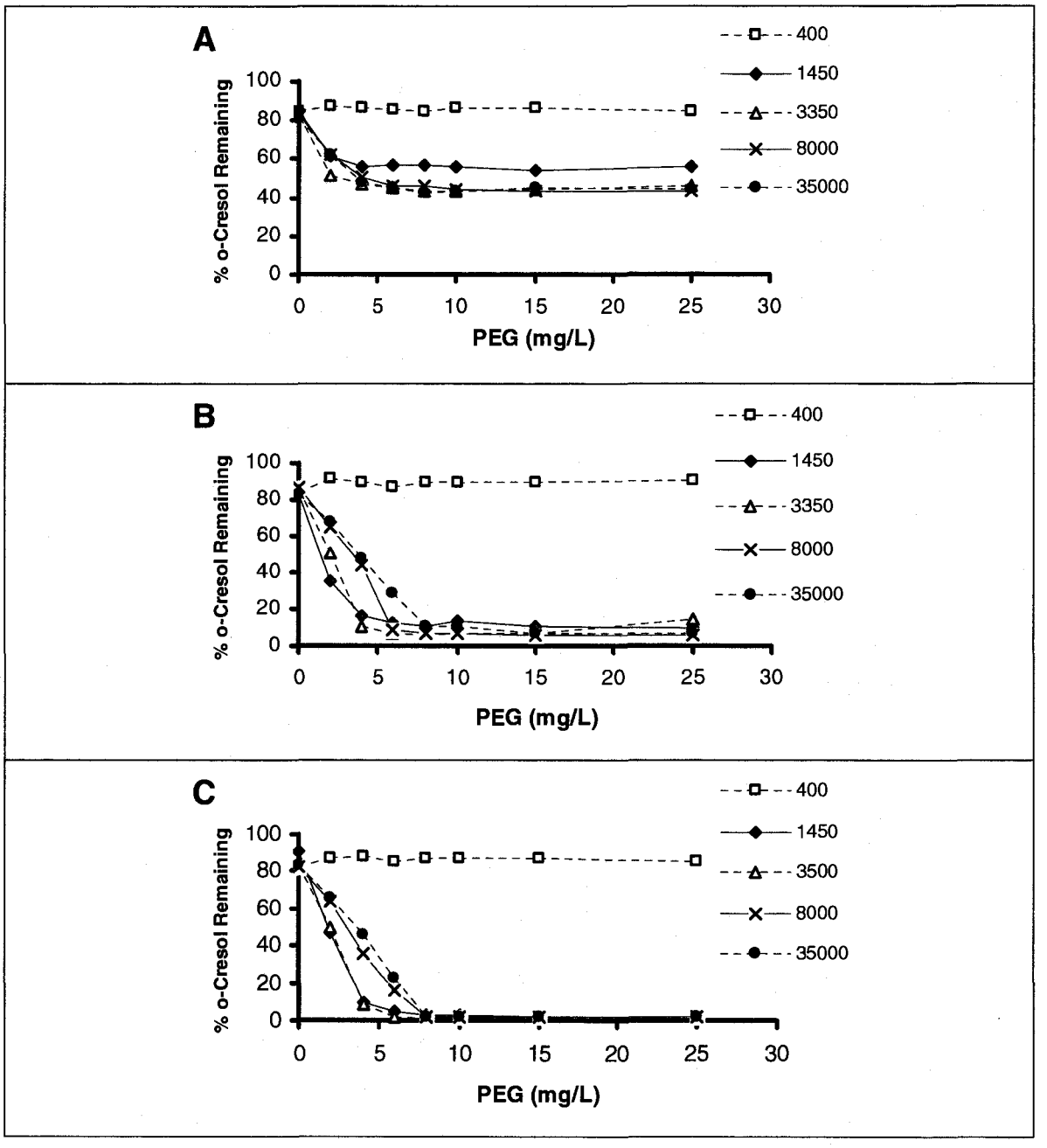


Figure 4.32: Molecular Weight Optimization for PEG with o-Cresol Narrow Range. (1.0 mM o-cresol; 3 h reaction (A), 14 h (B), 28 h (C); 40 mM acetate buffer, pH 5.68; 0.0015 LACU/mL; only analyzed by phenol colour test)

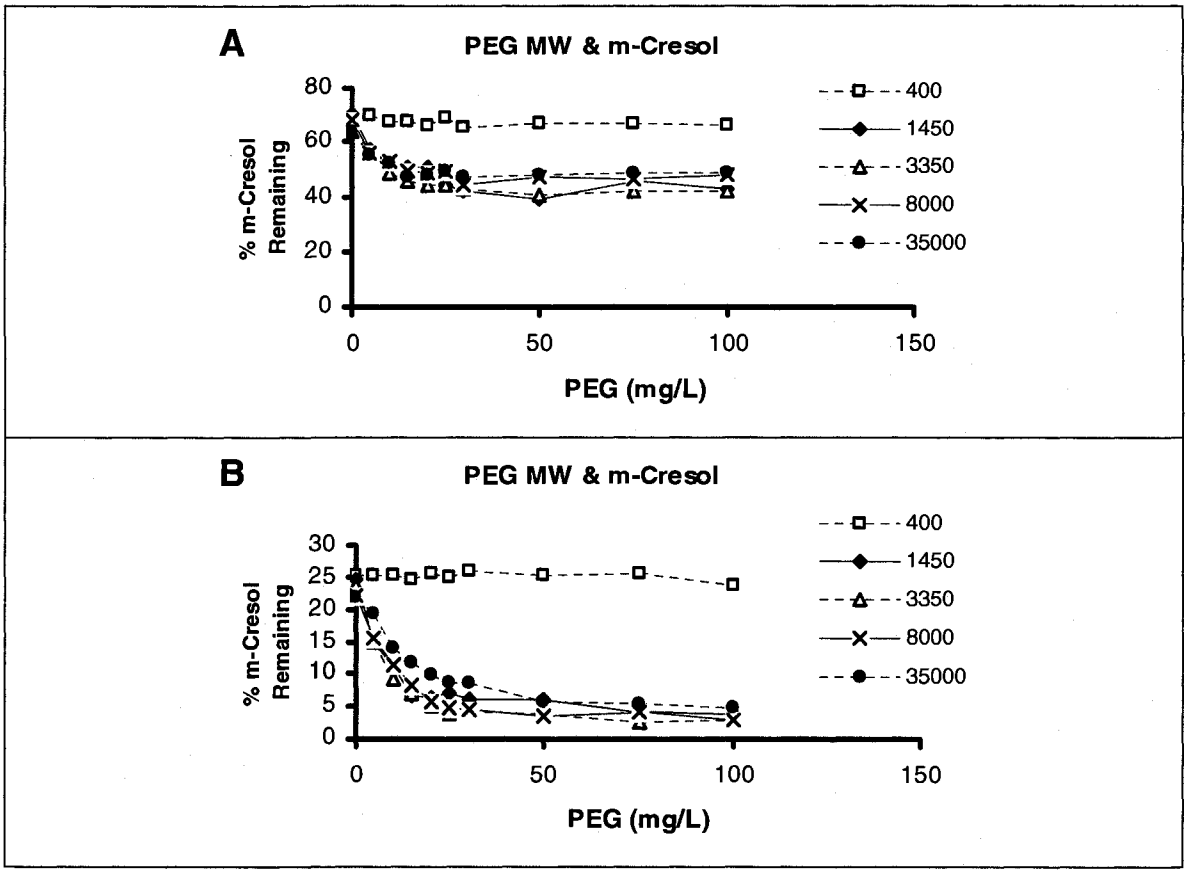


Figure 4.33: Molecular Weight Optimization for PEG with m-Cresol. (1.0 mM m-cresol; 3 h reaction; 40 mM acetate buffer, pH 5.68; 0.005 (A) 0.02 (B) LACU/mL; only analyzed by phenol colour test)

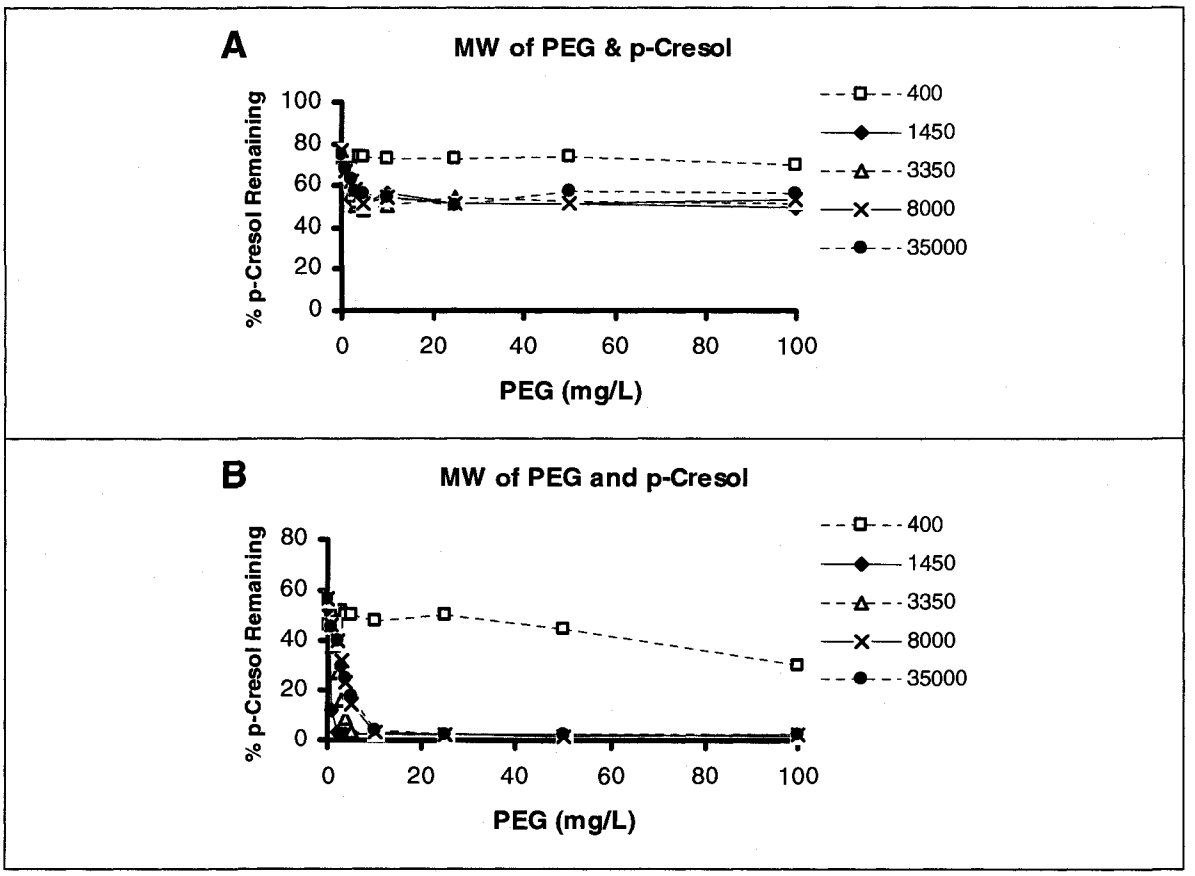


Figure 4.34: Molecular Weight Optimization for PEG with p-Cresol Broad Range. (1.0 mM o-cresol; 3 h reaction; 40 mM acetate buffer, pH 5.68; 0.0005 (A) 0.002 (B) LACU/mL; only analyzed by HPLC)

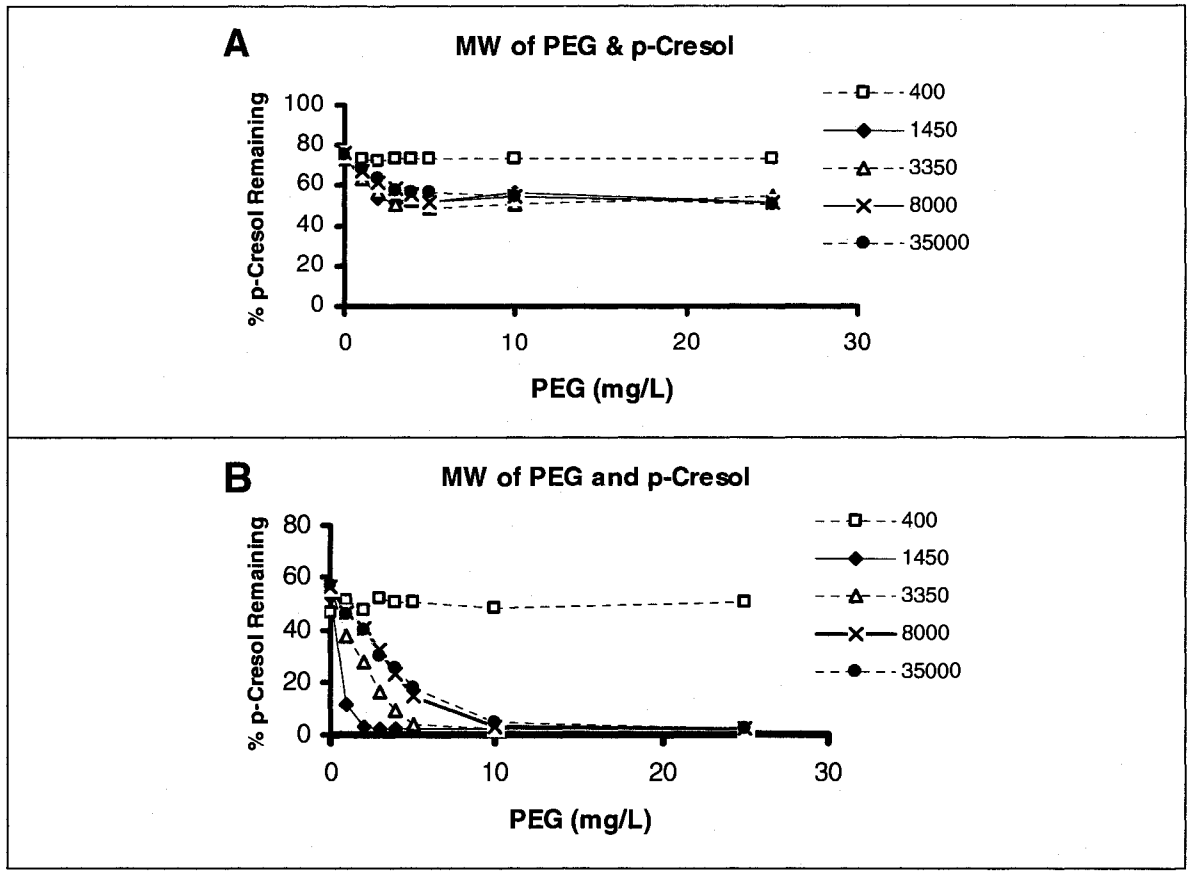


Figure 4.35: Molecular Weight Optimization for PEG with p-Cresol Narrow Range. (1.0 mM p-cresol; 3 h reaction; 40 mM acetate buffer, pH 5.68; 0.0005 (A) 0.002 (B) LACU/mL; only analyzed HPLC)

Table 4.7: Optimum PEG Concentrations for various MW's of PEG

	o-Cresol Optimum PEG mg/L		m-Cresol Optimum PEG mg/L		p-Cresol Optimum PEG mg/L	
PEG AVG MW	**3 h**	**28 h**	**3 h**	**24 h**	**3 h**	**24 h**
400	SC	SC	SC	ND	SC	SC
1450	4-6	6-8	25-30	ND	2-3	3
3350	4-6	6-8	25-30	ND	4-5	4-5
8000	6-8	8-10	30-50	ND	4-5	10
35000	6-8	8-10	30-50	ND	10	10

SC- same as control.

The results found in **Table 4.7** show some results in agreement with, as well as some that contradict previous studies. It was confirmed that PEG 400 (below 600) had no effect on conversion of any of the cresol isomers. It also appears as though higher concentrations

of PEG with larger MW's are needed and that their conversion efficiencies are slightly less efficient than those of the mid-range PEG's which contradicts previous studies (Saha 2006; Kinsely and Nicell, 2000). This does confirm some of the previous results with laccase (*Trametes versicolor*)-BPA studies in which the effectiveness of PEG increased with increasing MW up to 1000, had a slight optimum at 3350, then a slight decline in which BPA conversion stabilized (Kim and Nicell, 2006a). These results are different from *T. versicolor* in that PEG_{400} did not enhance removal efficiencies. The amount of PEG required was only optimized for PEG_{3350}, and it was not reported if more of the higher MW were required, or if the addition of more of the lower molecular weights could further enhance removal (Kim and Nicell 2006a). It was also found that, like the peroxidases and this study (**Fig. 4.23,4.25,2.27**), the optimum PEG concentration increased linearly with increasing substrate concentration (stoichiometric relationship) (Kim and Nicell 2006a) . Because it was observed that PEG's with MW below 1000 worked for BPA-laccase and steadily increased to 3350 then decreased, similar to this study (referring only to the decrease after 3350), while improved phenol conversion with SBP seemed to continue indefinitely with increasing PEG MW, may be evidence that two different types of protective mechanism are involved. From the o- and p-cresol in which studies longer than 3 h were done, it is quite evident that the enzyme is still catalytically active while PEG_{400} and the control had no significant conversion after the first 3 h. PEG_{400} also had no effect with laccase SP 504 and DPA (Saha 2006). This is evidence that PEG is capable of providing a protective effect on the catalytic oxidation of cresols by laccase SP 504. Similar results were reported with BPA polymeric products (Modaressi et al., 2005). Interestingly, borate has also been reported to have an

adsorption suppression effect but further studies were not done because of the possible environmental effect of releasing borate, which was optimized at 0.4 M for 10 g/L of phenol (Nakamoto and Machida, 1992). Borate has the capability to react with vicinal hydroxyl groups in a cis configuration to form borate complexes with polyhydroxyl compounds (Nakamoto and Machida. 1992). If this was the case it could be anticipated that PEG's with lower MW would have more H-bonding sites/mass unit and would be more effective at protecting the enzyme. PEG or PEO have unusually high solubility in water and the relationship between the size of the polymer chain, the water volume as well as structure have been studied extensively (Shikata et al., 2006; Aray et al., 2004). The number of hydrated water molecules (n) to ethylene oxide monomer units in PEO aqueous systems varies depending on the MW of polymer and the method used to calculate it ranging from 1-6 (Shikata et al., 2006; Kjellander and Florin, 1981; Huang and Nishinari, 2001). Hydration values for PEG 400, 1540, 70000 were reported as 1.6, 2.4, 3.3, respectively (Sato et al., 1998). It is thought that when the EO units reach 1500 the hydration value levels off at 3.7, in which case additional EO units have no effect (Shikata et al., 2006). From the results obtained in this study and others it appears as though the binding of water plays some effect in PEG's capacity to protect the enzyme. In prior studies other additives such as Dextran (MW-20,000 - 500,000), Protasan PG 210 (PRT; chitosan glutamate), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA; MW 100,000), DEAE-cellulose (DEC), biosoluble polymer© (BIP; copolymer of acrylamide, N- vinylpyrrolidone and ethyl acrylate), polyethyleneimine (PEI; MW 30,000-40,000), poly-L-lysine HBr (PLL; MW 6000-9000), diethylaminoethyl-dextran (DED; MW 500,000), ficoll (FIC; MW 400,000), polyacrylic acid (PAA), heparin (HEP), bovine

serum albumin (BSA), human serum albumin (HSA), milk casein, gelatin, and polyethylene glycol PEG (200-40,000) have been investigated for possible protective effects (Nakamoto and Machida, 1992; Kulys et al., 2003). HRP showed an adsorption suppression effect with milk casein, bovine serum, and PVA although quantitative data were not given (Nakamoto and Machida, 1992). With laccases a large number of polymers with various lengths, cationic, anionic and neutral charges have been examined (Kulys and Vidziumaite 2002; Kulys et al., 2003). From these studies it was concluded that polymer structure and possible interaction with enzyme may all be contributing factors. The laccases (rPpL – *Polyporus pinsitus*, rMtL *Myceliophthora thermophilia*) under investigation had pI values below 5.5 in which reactions were run, thereby imposing a net negative charge on the protein. Under these conditions cationic polymers seemed to inhibit the initial uptake of O_2 due to possible complexation with the negatively charged laccase. When a laccase (rCcL *Coprinus cinereus*) with a pI above 5.5 was used the addition of the same polymers had quite different effects (Kulys and Vidziumaite, 2002). Alginic acid, PAA, HEP, all of which are anionic and thought to form random coils in solution due to electrostatic repulsion with the negatively charged protein. These compounds showed no or little change in total oxygen consumption or initial rate of naphthol oxidation (Kulys et al., 2003). Other polymers showed a protective effect following BSA< D20 < PEG < PVP < D100 < D500 < HEC < BIP < FIC < PVA although PEG had a much greater protective effect on 2-napthol than 1-napthol, and vice-versa for PVP (Kulys et al., 2003). These polymers had similar initial O_2 consumptions but total O_2 consumption was increased. Considering the types of polymers used it is evident that their monomeric units have little in common but they are

all extremely water soluble and with increasing length form globular type complexes as hydration values increase. Thus it has been speculated that globular structure of polymers are required for the prevention of enzyme inactivation (Kulys et al. 2003). The fact that the cresols also had similar results with PVP of two MW's and Triton X-100 seems to support this theory (**Fig. 4.36-37**). All of the neutral polymers worked except the dextrans which were demonstrated to work with the napthols (Kulys et al. 2003). PEI, the only cationic polymer had a negative effect, similar to the napthols. When three polyelectrolytes were used with HRP, PERCOL LT24 (cationic), PERCOL LT20 (non-ionic), and Aquafloc 6465 (anionic) all showed an increase in standard activity assays of 10-25%, no explanation for this was presented but was confirmed by the increase in initial H_2O_2 consumption (Wu et al., 1998). PEG also showed a 10-20% increase on laccase activity assay (Modaressi et al., 2005). Various additives were tested with the three cresol-isomers at 5.0 mg/L and 50 mg/L to determine their effectiveness with Laccase SP504. PEG was the most effective at 5.0 mg/L, while PVP 1 (average MW of 10,000) was most effective for some of the isomers at 50.0 mg/L (**Fig 4.36-37**). Since it was the most effective at the lower concentration, PEG as an additive was further investigated. A more detailed investigation into the complexation of various intermediates is essential to clarify the structure–activity relationship.

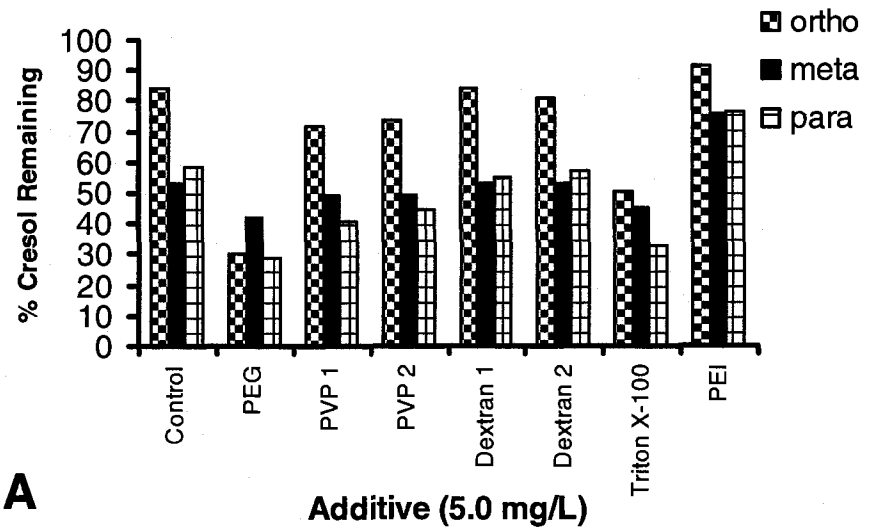


Figure 4.36: Removal of Cresols in the Presence of 5.0 mg/L of Various Additives. (Run at optimum pH in 40 mM phosphate buffer; 3 h; Control – no additives; PEG average MW 3350; PVP 1 average MW 10,000; PVP 2 average MW 40,000; Dextran 1 average MW 80,000; Dextran 2 average MW 282,000; PEI average MW 750 000, LACU/mL for o,m,p-cresol 0.002, 0.01, 0.001 respectively).

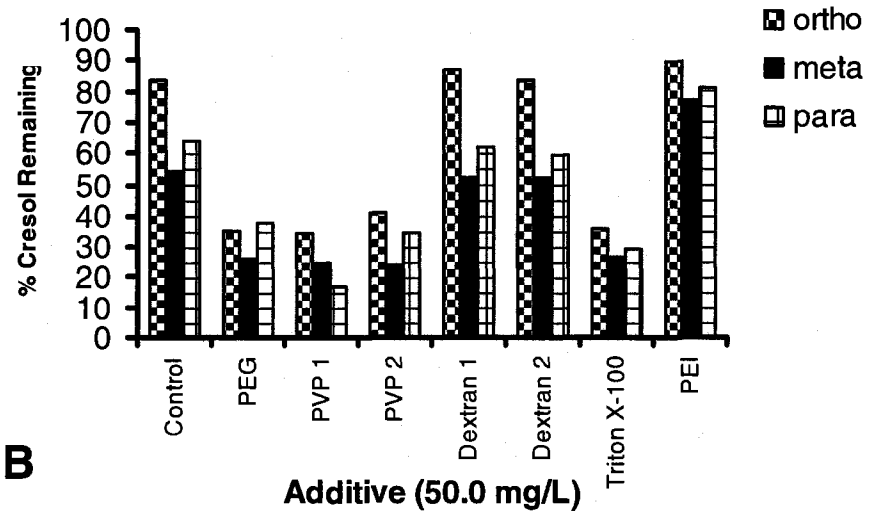


Figure 4.37: Removal of Cresols in the Presence of 50.0 mg/L of Various Additives. (Run at optimum pH in 40 mM phosphate buffer; 3 h Control – no additives; PEG average MW 3350; PVP 1 average MW 10,000; PVP 2 average MW 40,000; Dextran 1 average MW 80,000; Dextran 2 average MW 282,000; PEI average MW 750 000, LACU/mL for o,m,p-cresol 0.002, 0.01, 0.001 respectively).

4.5: Fate of PEG

Based on results of the previous section, PEG, in addition to having a protective effect on the enzyme is an ideal candidate for application because it has been FDA (Food and Drug Association) approved for use in injectable, topical, rectal, and nasal formulations, found in many over-the-counter pharmaceuticals and cosmetic products, with no adverse affects to humans (Treetharnmathurot et al., 2008). Currently no long term ecological effects of PEG have been cited. Besides being very inexpensive it has shown to have little negative overdose effect, shown in some cases by gelatin and some polyelectrolytes (Wu et al., 1997). When using any synthetic polymer the effects of releasing it with the effluent should be minimized due to its potential environmental impacts such as oxygen demand. Otherwise, a cheap, efficient strategy for removal before the effluent release should be included in the treatment operations (Kinsely and Nicell, 2000). TOC (total organic carbon) analysis on supernatants was done to determine residual PEG after enzymatic treatment. Reactors with 1.0 and 5.0 mM o-cresol and 1.0 and 3.0 mM m,p-cresol were run in 40 mM phosphate buffer at pH 5.7 with increasing PEG from 0- 250 mg/L depending on isomer and initial substrate concentration. After the 3 h reaction periods the samples were acid quenched below pH 2 with H_2SO_4 and left to sit overnight before being microfiltered. Residual PEG is determined by taking the total TOC – (IC inorganic carbon) – (TOC of residual substrate remaining, predetermined by HPLC analysis). A control with just buffer and enzyme was run in each case and it was determined that the TOC contributed by the enzyme in solution was negligible and therefore excluded from the calculation. Results are plotted in **Fig. 4.38-43**. All reactions achieved > 90% removal after PEG requirement was met; similar conversions were desired so that TOC due to

polymeric products were of similar concentration. All samples were run in duplicate. The only set of reactors where significant conversion was not achieved was the 5.0 mM o-cresol set despite using the equation generated in section **4.2**. This was not anticipated and the inference is that the higher oxygen demand due to higher o-cresol concentrations may be limiting and caused deviations from the prior trend in section 4.2 which was only examined from a range from 0.25 to 2.0 mM. It can be observed in **Fig 4.38** and **4.39** that when no PEG (0 mg/L) was added and lower conversion is achieved the TOC values were significantly higher despite the subtraction of the residual o-cresol, most likely due to a higher concentration of soluble products. A PEG monomeric unit of (EO) OCH_2CH_2 is approximately 54.5% carbon by mass. Based on this assumption the theoretical accumulation of PEG in supernatant should be linear with a slope for TOC of 0.545 mg/L and 1.0 mg/L for PEG.

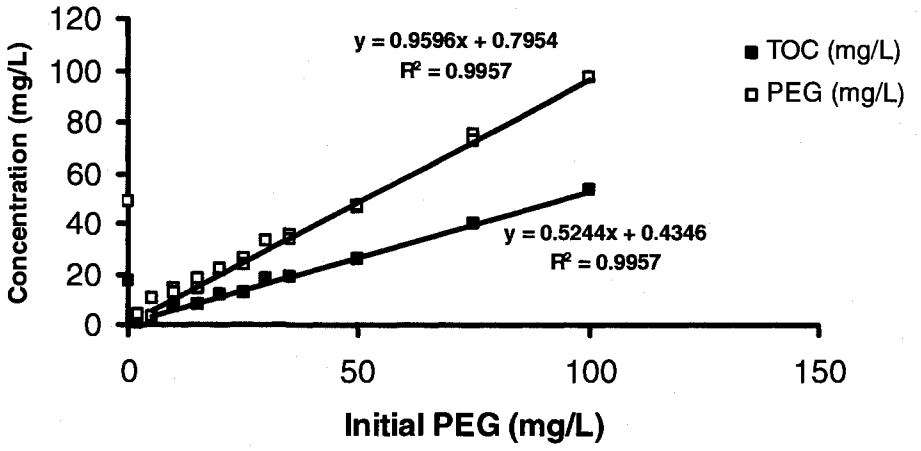


Figure 4.38: TOC of Supernatant Following Enzymatic Treatment and Microfiltration as a Function of PEG Dose with 1.0 mM o-Cresol. (run with phosphate buffer pH 5.7; 0.008 LACU/mL; for 3 h; quenched with acid > pH 2; micro-filtered; TOC run 24-48 h after quenching; residual TOC from o-cresol subtracted).

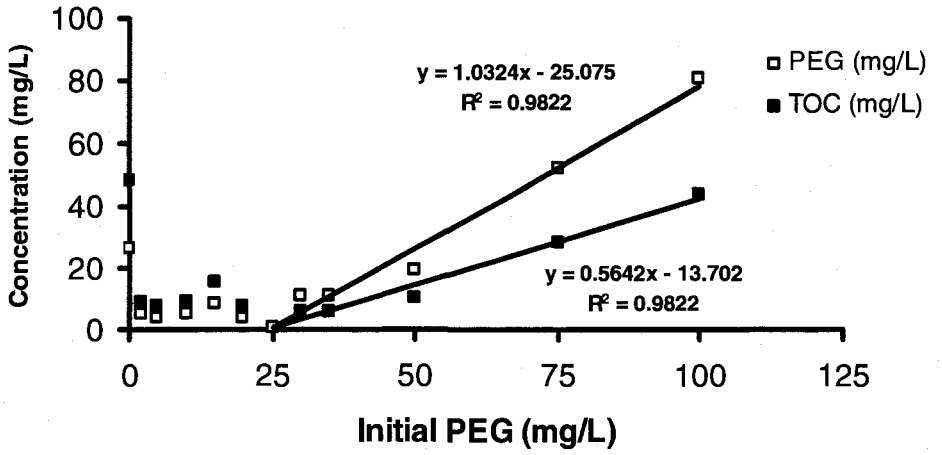


Figure 4.39: TOC of Supernatant Following Enzymatic Treatment and Microfiltration as a Function of PEG Dose with 5.0 mM o-Cresol. (run with phosphate buffer pH 5.7; 0.0175 LACU/mL; for 3 h; quenched with acid > pH 2; micro-filtered; TOC run 24-48 h after quenching; residual TOC from o-cresol subtracted).

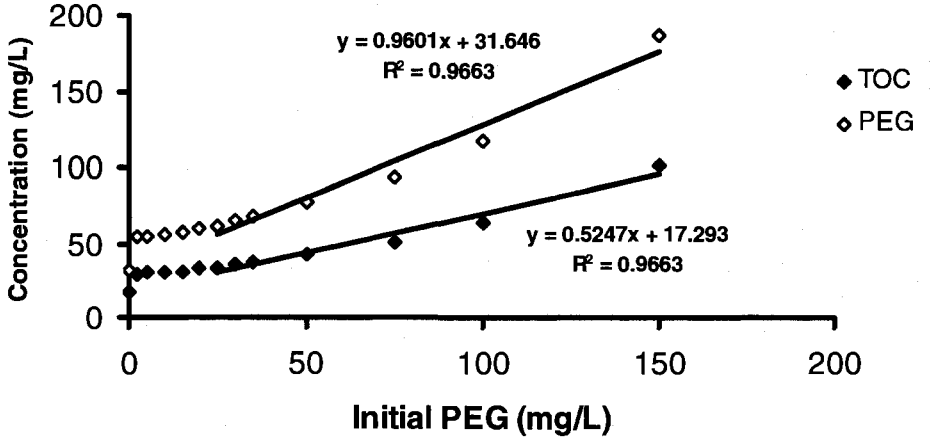


Figure 4.40: TOC of Supernatant Following Enzymatic Treatment and Microfiltration as a Function of PEG Dose with 1.0 mM m-Cresol. (run with phosphate buffer pH 5.7; 0.05 LACU/mL; for 3 h; quenched with acid > pH 2; micro-filtered; TOC run 24-48 h after quenching; residual TOC from m-cresol subtracted).

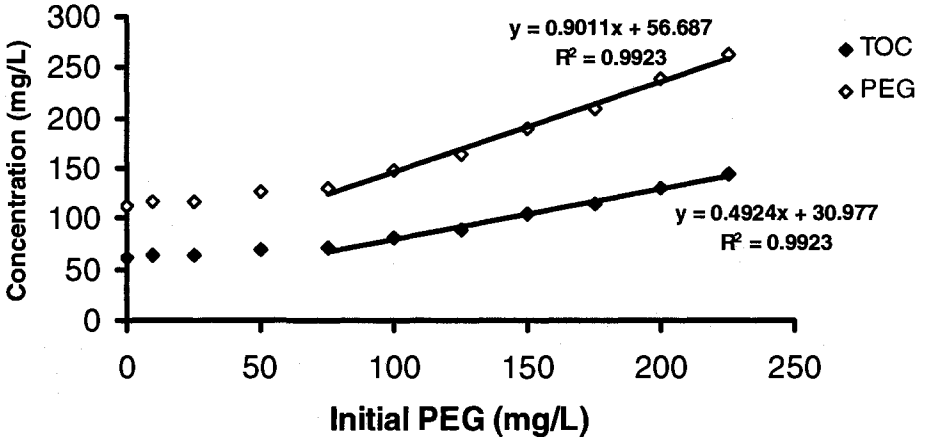


Figure 4.41: TOC of Supernatant Following Enzymatic Treatment and Microfiltration as a Function of PEG Dose with 3.0 mM m-Cresol. (run with phosphate buffer pH 5.7; 0.12 LACU/mL; for 3 h; quenched with acid > pH 2; micro-filtered; TOC run 24-48 h after quenching; residual TOC from m-cresol subtracted).

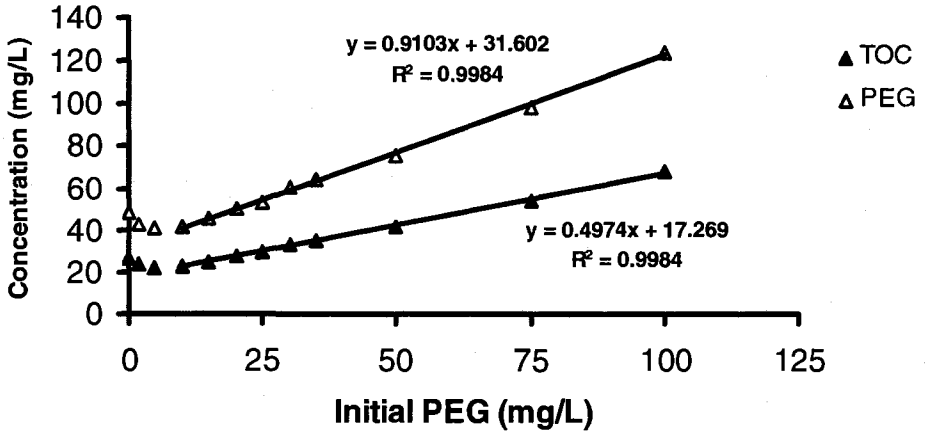


Figure 4.42: TOC of Supernatant Following Enzymatic Treatment and Microfiltration as a Function of PEG Dose with 1.0 mM p-Cresol. (run with phosphate buffer pH 5.7; 0.002 LACU/mL; for 3 h; quenched with acid > pH 2; micro-filtered; TOC run 24-48 h after quenching; residual TOC from p-cresol subtracted).

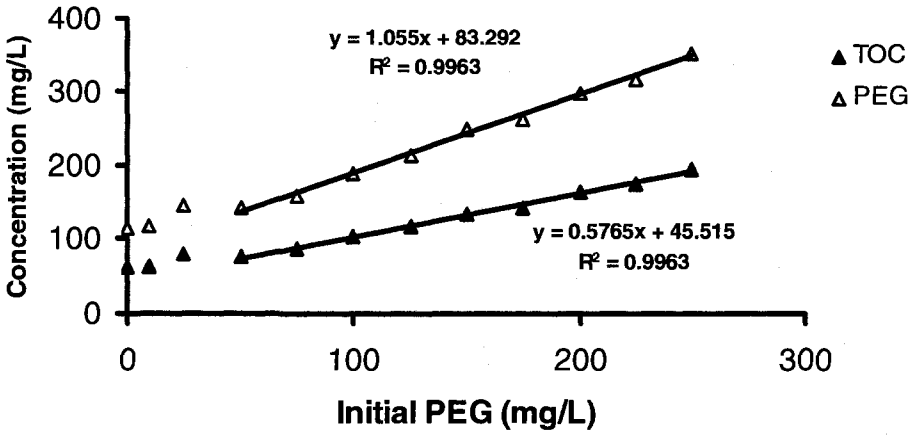


Figure 4.43: TOC of Supernatant Following Enzymatic Treatment and Microfiltration as a Function of PEG Dose with 3.0 mM p-Cresol. (run with phosphate buffer pH 5.7; 0.0035 LACU/mL; for 3 h; quenched with acid > pH 2; micro-filtered; TOC run 24-48 h after quenching; residual TOC from p-cresol subtracted).

Table 4.8: Summary of TOC Results from Fig. 4.38-43						
	o-Cresol		m-Cresol		p-Cresol	
		Predicted value[#]		Predicted value[#]		Predicted value[#]
PEG (mg/L) accumulation starts for 1 mM	5	4.4	25	18.0	10	5.4
PEG (mg/L) accumulation starts for 3 mM	25*	14.8*	75	72.5	50	13.0
Slopes for TOC and PEG (mg/L)						
	TOC	**PEG**	**TOC**	**PEG**	**TOC**	**PEG**
1 mM	0.5244	0.9596	0.5247	0.9601	0.4974	0.9103
3 mM	0.5642*	1.0324*	0.4924	0.9011	0.5765	1.055

* 5 mM initial substrate;
[#] Predicted Values based on linear equations in **Table 4.6** not TOC values.

In theory if PEG was attaching (or any type of interaction) to the phenol polymeric products (polyphenols) responsible for inactivating the enzyme, any excess PEG should be found in the supernatant. If this were the case it would be expected that the optimum PEG concentration should correlate to the amount of PEG associated with these polymers and therefore also be removed by micro-filtration. In this case it is observed that more than the optimum PEG concentration is being precipitated along with the insoluble polymeric products. There were larger jumps in PEG concentrations in the TOC study and the point of accumulation may possibly be overestimated. For example, **Table 4.8** lists 50 mg/L of PEG as the threshold at which PEG starts to accumulate in the supernatant; however there was a jump from 25-50 mg/L in which accumulation may have already started thus skewing the predicted threshold for PEG accumulation. Results for m-cresol seem to correlate decently between PEG optimization studies but deviations with o- and p-cresols seem to increase with increasing substrate concentration. When examining the linear portions of **Fig 4.38-43** listed in **Table 4.8** with values for both TOC

mg/L and its corresponding value for PEG mg/L we see that the slopes for 1.0 and 5.0 mM o-cresol, 1.0 mM m-cresol, and 3.0 mM p-cresol were within 5.0% of the theoretical value of TOC for PEG (TOC = 0.54 mg/L or PEG = 1.0 mg/L) while 3.0 mM m-cresol and 1.0 mM p-cresol slopes predicted values within 5 to 10% of the theoretical value. It is anticipated that if more than duplicate reactors were run for each sample that the error would be reduced. Standard curves with o-cresol and PEG were run with experimental values within 3.0% of theoretical values. Despite the larger than anticipated error, the R^2 values for **Fig. 4.38-43** are all between 0.9663 – 0.9984 which indicate a definite linear trend towards organic carbon accumulating in supernatant with increasing PEG concentration. In previous studies it was reported that 23% of (35 mg/L) $PEG_{35\ 000}$ remained in solution with 1.0 mM phenol and SBP (Kinsley and Nicell, 2000) and 30 % of (4.0 g/L) PEG_{1000} remained in solution when treating high concentrations of phenol with HRP (10 g/L) (Nakamoto and Machida, 1992). However, Wu et al., (1997) reported that all of the PEG_{3350} used to treat 1.0 mM phenol with HRP precipitated with the polymeric products, when added at the minimum effective concentration. With HRP and SBP used for the treatment of 1.0-10.0 mM phenol it was determined that the soluble residual products were insignificant when determining the COD and thus for the peroxidase studies the remaining TOC (COD) is deemed as the residual PEG concentration remaining in solution (Ghioureliotis and Nicell, 1999). Residual TOC in **Fig 4.38-43** is assumed to be both PEG and soluble polymer. With o-cresol this was confirmed in that the supernatant retained a greenish colour after micro-filtration with a λ_{max} in the visible range 404-420 nm as well ~ 270 nm both of which PEG does not exhibit.

o-Cresol products made from 1.0 mM were filtered on nitrocellulose membranes, and dried in a desiccator for several days. These products were then centrifuged at 8000 rpm for 5-10 min and the supernatant analyzed for TOC. Two washes produced no TOC suggesting that the PEG was not being washed off the products. The fact that PEG is not washed off the products suggests possible covalent interaction with PEG. In hindsight a higher concentration of initial substrate or m-cresol where the amount of PEG retained is higher should have been used to confirm results. A similar study done with HRP and phenol found that the PEG could not be washed off the polymeric products (Wu et al., 1998). The precipitate made from HRP-phenol and HRP-phenol-PEG were both incubated with enzyme and activity assessed over time. The precipitate that was made with PEG no longer inactivated the enzyme while the precipitate made without PEG inactivated the enzyme. Furthermore, when HRP was incubated with PEG it was protected from inactivation by the HRP-phenol precipitate. This proves that the inactivation of peroxidases still occur by means other then phenoxyl radical attack on the active site, but does not disprove any inactivation due to radical attack (Wu et al., 1998). It also confirms that the polymeric products generated are a significant source of inactivation. Consistent with the peroxidase studies, laccase products formed without PEG are darker and finer than when PEG is used (Wu et al., 1998). The fact that PEG seems to be retained in the products is a desirable attribute for an additive because it eliminates the additional step in the process of removing it before effluent release.

4.6.: Activity Over Time

4.6.1 SP 504

To confirm that PEG has the capability of protecting laccase SP 504 from inactivation, enzyme activity through the course of the reaction was monitored. Batch reactors were run in a minimum of triplicate due to the sensitivity of the activity assay and large dilution factors. Control reactors with only reactant and syringaldazine showed that none of the initial reactants interfered with the assay under the same time scale and conditions. There is no way to really test the possible interference of the reacting intermediates. Therfore the preceeding study in which removal over time was monitored was done to corroborate these results (**Section 4.7**). Despite increased trials substantially larger than 5.0% deviation for most data points was observed and so the error bars for these studies were included. A control reactor was run with the same initial laccase concentration, buffer, with and without additive, mixed and non-mixed and no substrate to determine the amount of inactivation under non-reacting conditions. At most, a loss of 8.0% activity (with controls) was observed over the three-hour reaction period and could be subtracted from the reaction inactivation conditions. Reactors for substrate were used with optimum laccase, at optimum pH, with 1.0 mM substrate, run for 3 h. Activity was taken with filtered and non-filtered samples to discern if active enzyme was being adsorbed onto the polymeric products. Results are given in **Fig 4.44-46** and **Table 4.9** and laccase activity for phenol conversion over time is plotted in **Fig 4.54** and **4.74** (wastewater).

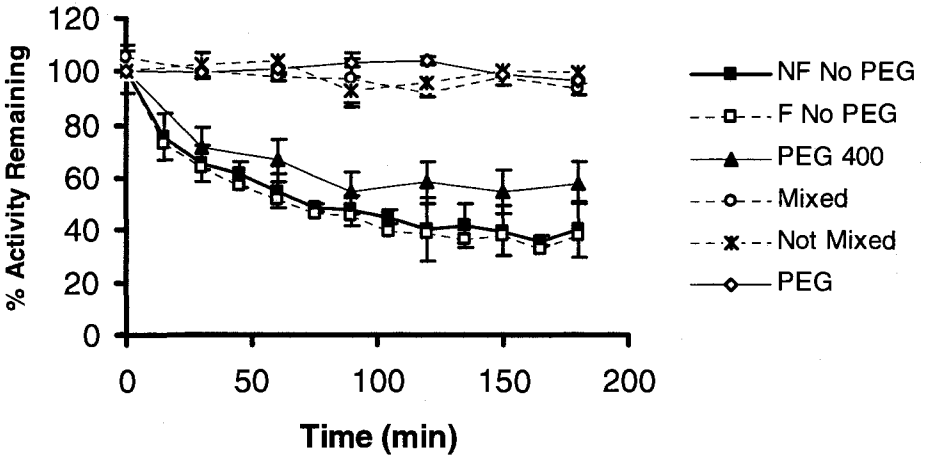


Figure 4.44: Activity of Laccase with 1.0 mM o-Cresol. NF- Not Filtered, F – Filtered, Mixed and non-mixed reactors were run as controls under the same condition without o-cresol added (Non-reacting conditions). (3 h reactions; 40 mM phosphate or acetate buffer at pH 5.6; 0.045 LACU/mL)

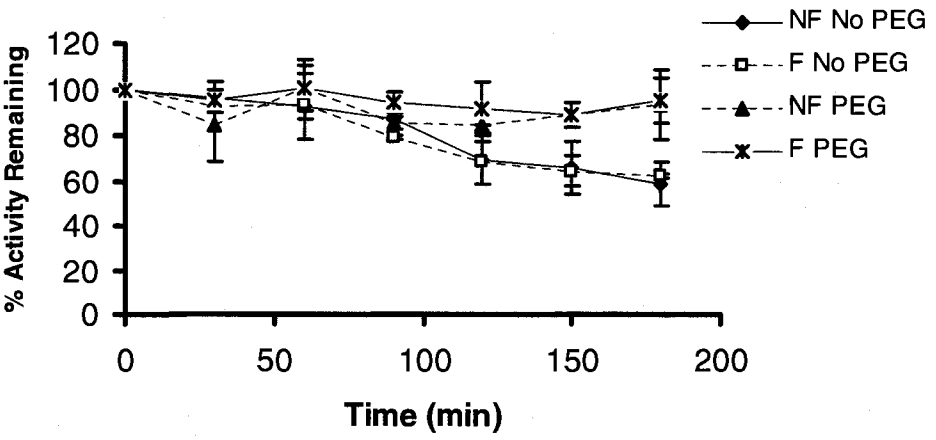


Figure 4.45: Activity of Laccase with 1.0 mM m-Cresol. NF- Not Filtered, F – Filtered, (3 h reactions; 40 mM acetate buffer at pH 5.6; 0.045 LACU/mL)

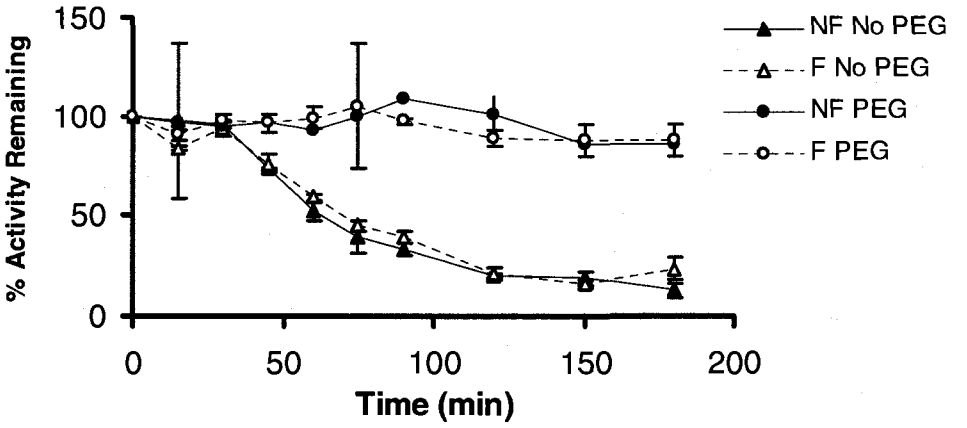


Figure 4.46: Activity of Laccase with 1.0 mM p-Cresol. NF- Not Filtered, F – Filtered, (3 h reactions; 40 mM acetate buffer at pH 5.6; 0.007 LACU/mL)

Table 4.9: Average Loss of Activity for Phenolic Substrates

	C_1 Not Stirred (Non-reacting) (%)	C_2 Stirred (Non-reacting) (%)	Phenol (%)	o-Cresol (%)		m-Cresol (%)	p-Cresol (%)
PEG	ND	2-5	ND	2-4	44-50*	4-7	6-12
No PEG	5-8	2-5	10-14	60-68		38-45	80-88

ND – Not Done, *PEG 400, phenol results in **Fig 4.54**.

The results obtained with phenols give further evidence to support the "sacrificial polymer theory" for the protective effect of PEG. When comparing the results we find that phenol, which had no "PEG effect," only had a loss of 10-14% activity, while all of the cresol isomers have significantly higher levels of inactivation ranging from 38-88% in the absence of PEG. As expected when o-cresol was incubated with PEG_{400} no protective effect was observed despite a slightly lower level of inactivation. The degree of inactivation varies depending on the cresol isomer and is consistent with the progress of visible polymer formation in the batch reactors. o-Cresol reactions have the majority of inactivation in the first 60 min, when a large flake-like precipitate forms quite quickly.

p-Cresol reactions have the majority of inactivation in the first 100 min, the polymeric products are white in colour and much finer then o-cresol solids. m-Cresol reactions have very little inactivation in the first 100 min, and in these reactors solid precipitate is usually not observed within the first hour although the reaction mixture turns a tan colour. The fact that inactivation of m-cresol takes so long may account for its smaller PEG effect previously discussed. Enzyme activity with BPA and laccase SP 504 was relatively stable for the first 2 h (loss of ~ 10%) with a rapid decline to complete inactivation at 3 h (Modaressi et al., 2005). Formation of solid precipitates is not a requirement for inactivation. Even at lower enzyme concentrations where no precipitate is visible, laccase activity is reduced, as is the case with peroxidases, but such products are thought to be less inactivating than the more mature products (Wu et al., 1998). Samples that had been microfiltered showed similar activity changes to the unfiltered samples. This implies that the free enzyme either adsorbs to products smaller then those removed by filtration or not at all, also observed in Modaressi et al., (2005). In comparison, the HRP-phenol reactions showed a maximum of 30 % HRP activity adsorbed onto the solid product (Wu et al., 1998). Generally it is observed that peroxidases are inactivated much quicker the laccases; in the case of SBP it may only be a matter or minutes (Steevensz et al., 2008).

4.6.2 Laccase SP 805

Prior to this study laccase SP 805 was investigated in this lab for the removal of cresols (Vermette 2000). In that study it was found that PEG had no effect on the removal of any of the isomers. Activity monitored over time demonstrated that laccase SP 805 had less than 10% loss in a 3 h reaction period. o-Cresol at two different enzyme concentrations

was run as a control with both the activity and removal monitored. All studies with laccase SP 805 were run under optimum conditions, pH 7.2-7.4, 40 mM phosphate buffer, previously determined by Vermette (2000), in which o- and m-cresols differed from p-cresol which had an optimum pH of 5.6. It was found that no activity was lost over the first 3 h and that removal was slow, as is observed with phenol and laccase SP504 (**Fig 4.47.54**). To further confirm this, PEG was added in increasing concentrations from 0-400 mg/L to batch reactors and run at optimum conditions for both o- and m-cresol (**Fig 4.48-49**). No PEG effect was observed. Consistent with previous studies no PEG effect is observed if the enzyme is not significantly inactivated. Removal was also monitored after 24 h reaction periods, again to accentuate the PEG effect for conditions were inactivation may be slow. No PEG effect was observed (**Fig 4.48-49**).

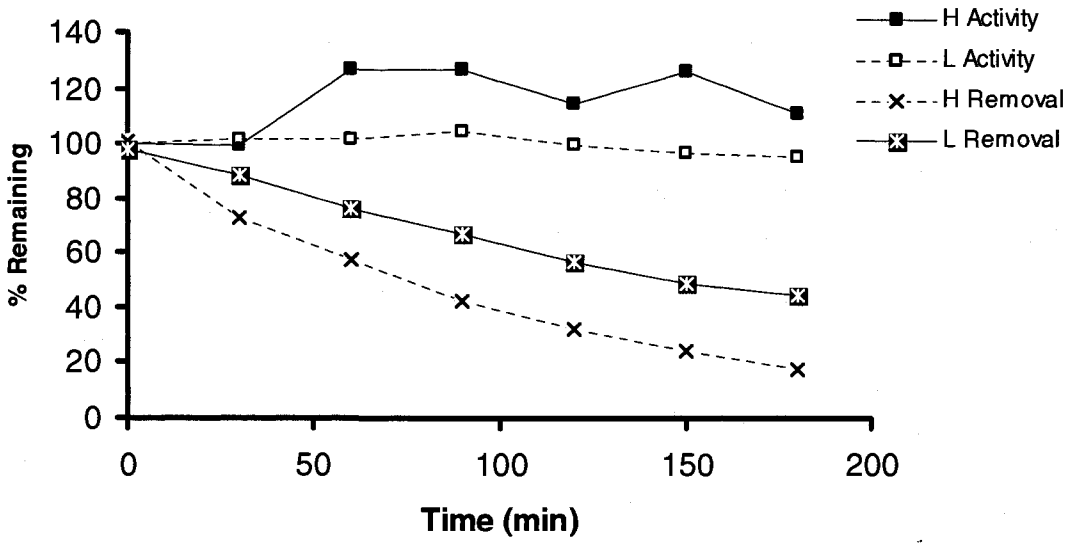


Figure 4.47: Activity and Removal Over Time Using Laccase SP 805 with 1.0 mM o-Cresol. (3 h reactions; 40 mM phosphate buffer, pH 7.2-7.4; 11.7 LAMU/mL, H- high, 2.55 LAMU/mL, L- Low).

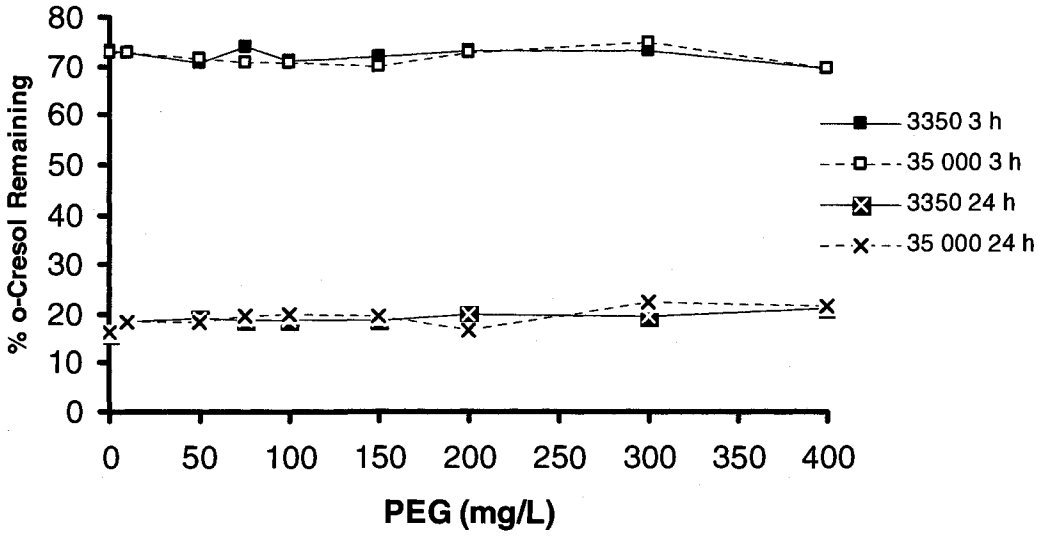


Figure 4.48: o-Cresol (1.0 mM) Conversion Using Laccase SP 805 with PEG. (3 h reaction; 40 mM phosphate buffer, pH 7.2-7.4; 1.11 LAMU/mL; analyzed only by colorimetric assay)

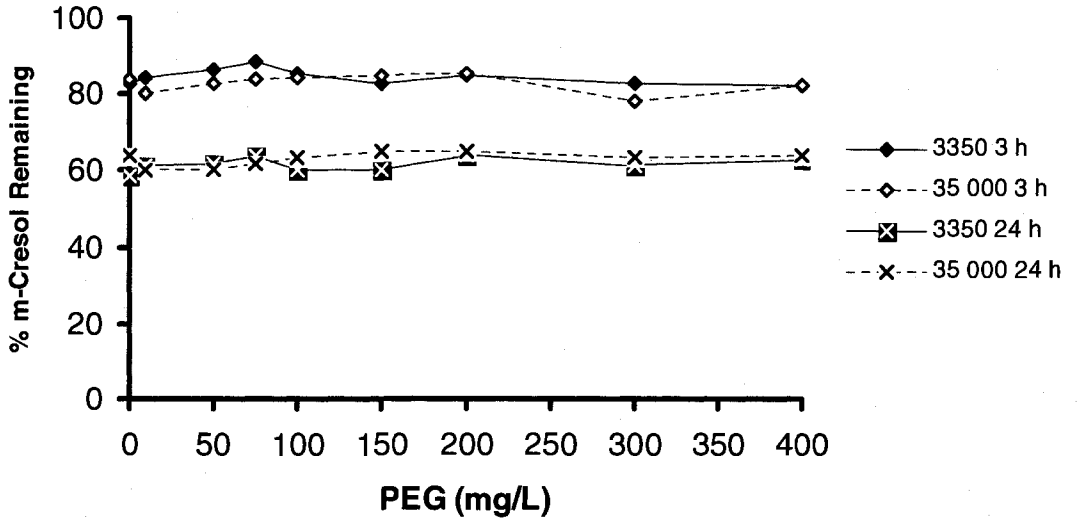


Figure 4.49: m-Cresol (1.0 mM) Conversion Using Laccase SP 805 with PEG. (3 h reaction; 40 mM phosphate buffer, pH 7.2-7.4; 1.11 LAMU/mL; analyzed only by colorimetric assay)

4.7: Removal Over Time

The reaction time chosen for these studies was 3 h, which is an arbitrary time used to compare enzymes and substrates being studied in our lab. Reaction time is one of the main parameters that are considered when designing a treatment plan because it is directly related to the reactor size which is one of the major economic considerations. It has been observed that peroxidases are much faster in oxidizing substrates than laccases (Steevensz et al., 2008). Since retention time of a reactor dictates the reactor volume it is useful to know the minimum time needed for $\geq$ 95 % conversion of pollutant. This can be optimized to various times but in this study only monitored for the pre-determined minimum enzyme concentration for $\geq$95 % conversion of the initial substrate concentration. In these studies, reactors were prepared with 1.0 mM substrate, run at optimum pH, with addition of a slight excess to the optimum PEG concentration when required, and with laccase at both optimum and limiting concentrations to corroborate residual activity findings. Aliquots were withdrawn at various time intervals, quenched with H_2SO_4 and analyzed by HPLC. Results can be found in **Fig 4.50-53**.

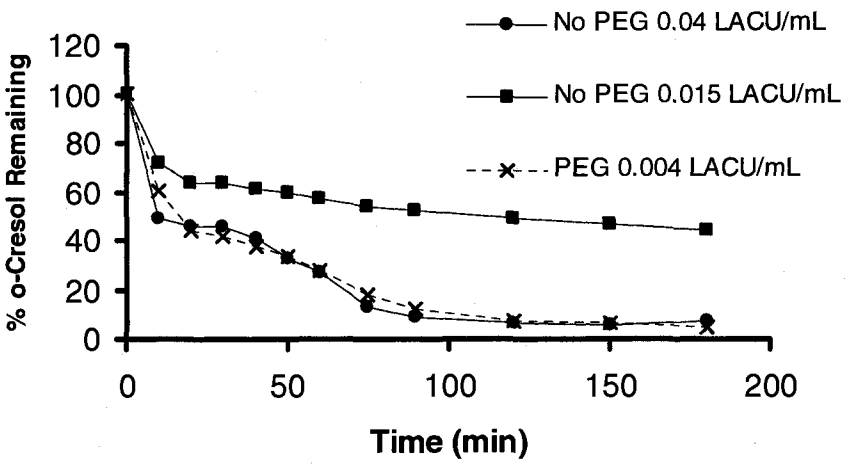


Figure 4.50: Removal Over Time for o-Cresol. (5.64 acetate buffer; 1.0 mM o-cresol; 3 h reaction; 25 mg/L of PEG when applicable)

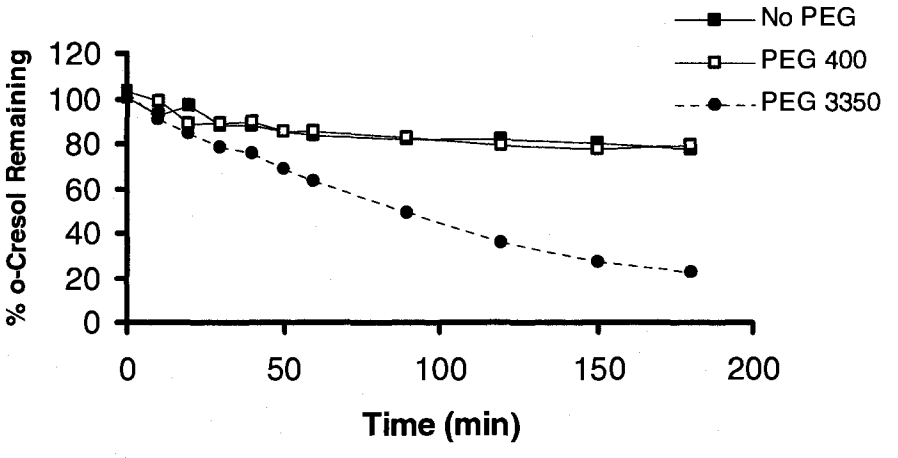


Figure 4.51: Removal Over Time for o-Cresol. (5.64 acetate buffer; 1.0 mM o-cresol; 3 h reaction; 25 mg/L of PEG when applicable; 0.0025 LACU/mL for all reactors)

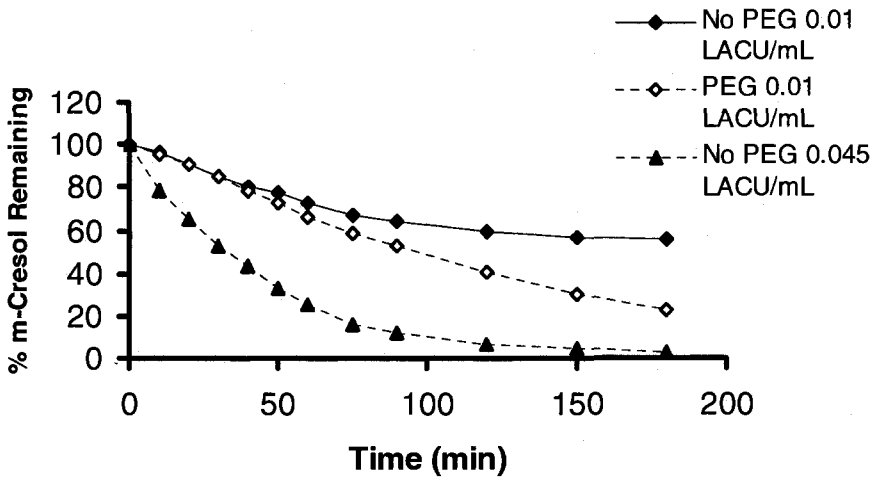


Figure 4.52: Removal Over Time for m-Cresol. (5.68 phosphate buffer; 1.0 mM m-cresol; 3 h reaction; 50 mg/L of PEG when applicable)

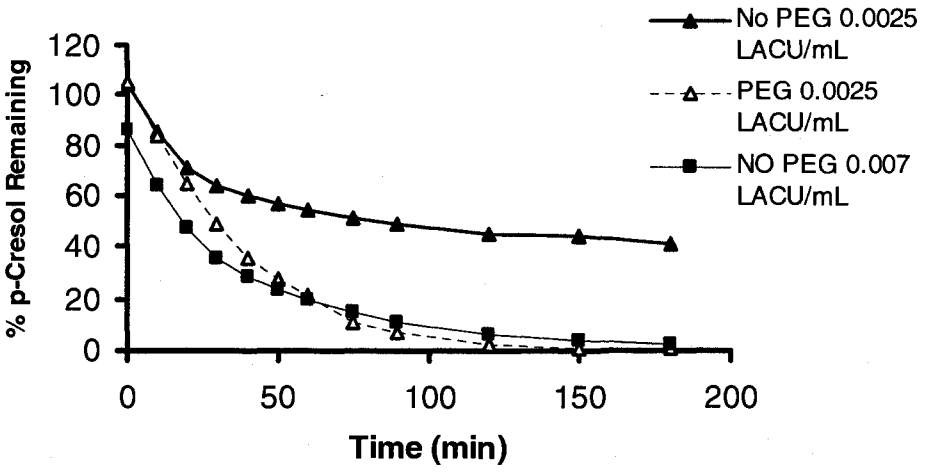


Figure 4.53: Removal Over Time for p-Cresol. (5.62 acetate buffer; 1.0 mM p-cresol; 3 h reaction; 50 mg/L of PEG when applicable)

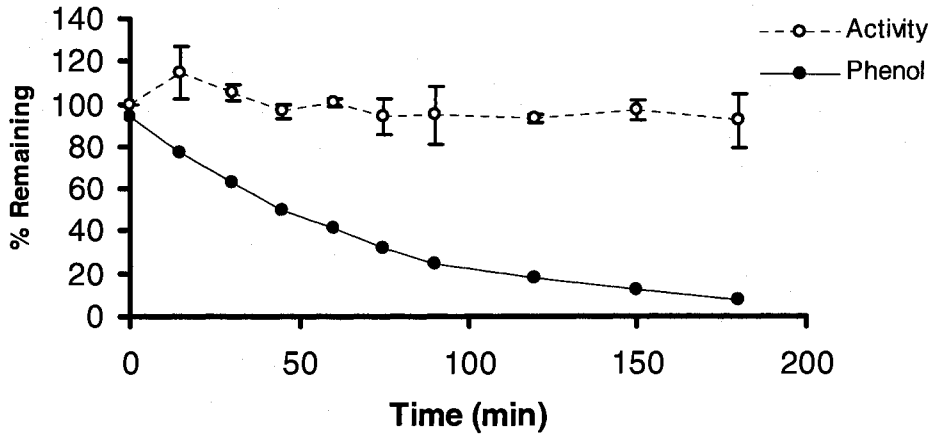


Figure 4.54: Activity and Removal Over Time for phenol. (5.7 phosphate buffer; 1.0 mM phenol; 3 h reaction; 0.08 LACU/mL)

In all cases when PEG is not added, the phenols are reduced to ≤ 20 % of the initial substrate concentration in the first 100 min if ≥ 95% removal is to be attained; most likely due to inactivation of the enzyme. Two factors are expected to slow the conversion of substrate as the reaction proceeds, the first being the inactivation of the enzyme observed in the preceding section and the second being the decrease in substrate concentration as the reaction proceeds. In **Fig 4.51-53** reactions with PEG and without PEG at the same enzyme concentration are shown. Conversion of the cresols levels off as the inactivation sets in. When the additive is present the conversion is observed to be much more consistent and since the reaction is slow the first-order decay is stretched so that it looks more linear (especially **Fig 4.51-52**), which is what would be expected if little activity is lost, and conversion rates only decreased due to the limiting availability of substrate. For the removal of phenol (**Fig 4.54**) it is evident that the entire 3 h reaction period is needed for ≥ 95 % removal, despite the low level of enzyme inactivation. SBP demonstrated that under optimum conditions (for 3 h reaction periods) for the removal of 1.0 mM aryl diols catechol, resorcinol, and hydroquinone, 60-85 % conversion in the first 15 min was

achieved and that 1.0 mM of aryl amines o-, m-, o,p- phenylenediamine had 65-95% conversion in the same time period (Mousa 2008). The rapid conversion of substrate is needed if significant conversion of the substrate is to occur due to the rapid inactivation of the enzyme (Wu et al., 1998). Increasing the enzyme concentration was shown to increase the rate of reaction but the level of inactivation increased proportionally, decreasing the turnover (Wu et al., 1999; Bodalo et al., 2006). Retention time in the reactor can be compensated by simply adding more or less enzyme depending on the substrate concentration.

For anilines, it is evident from **Fig. 4.55-56** that the enzyme in the batch reactors is still active due to the high conversion of a second aliquot of substrate. Aniline shows a decrease in conversion from the first 180 min to the second 180 min of ~ 25 % without PEG and increase of 17 % with PEG, most likely because those reactors had spiked concentrations that exceeded the original 1.0 mM concentration. p-Toluidine has a decrease in conversion rate of ~ 25 % when comparing the first 180 min of the reaction and the second 180 min with and without PEG. Since the aniline compounds have such a high enzyme requirement there is still a lot of free enzyme in solution. This may be why the PEG effect is not observed. As stated before, it is odd that no PEG effect is observed for any of the aniline compounds even with the peroxidases with the exception of one case involving DPA and laccase. The functional group and the intermediates likely play an important role in the PEG effect but little information on these intermediates is available.

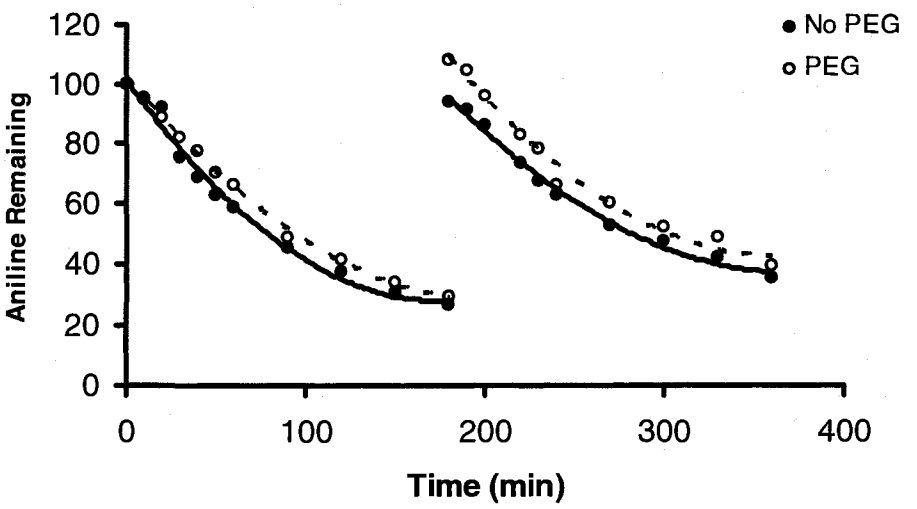


Figure 4.55: Removal of Aniline Over Time (1.0 mM aniline run at optimum pH, 40 mM buffer; 0.6 LACU/mL; after 3 h spiked back to near 100% of original substrate concentration, no addition of enzyme; No PEG, 1st 180 min $y = 0.0024x^2 - 0.8464x + 101.37$ $R^2=0.9883$; 2nd 180 min $y = 0.0016x^2 - 1.181x + 256.54$ $R^2 = 0.9922$; With PEG, 1st 180 min $y = 0.0017x^2 - 0.7175x + 101.86$, $R^2 = 0.9978$, 2nd 180 min $y = 0.002x^2 - 1.4559x + 305.88$, $R^2 = 0.9856$.

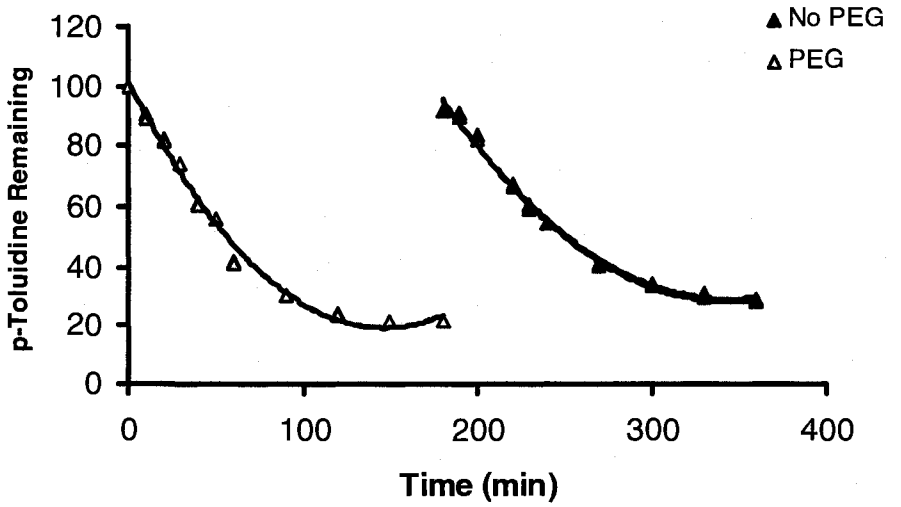


Figure 4.56: Removal of p-Toluidine Over Time (1.0 mM p-toluidine run at optimum pH, 40 mM buffer; 0.08 LACU/mL; after 3 h spiked back to near 100% of original substrate concentration, no addition of enzyme; No PEG, 1st 90 min $y = 0.0038x^2 - 1.1201x + 101.15$ $R^2 = 0.9924$; 2nd 90 min after spiking, $y = 0.0024x^2 - 1.6823x + 318.86$, $R^2 = 0.9932$; With PEG, 1st 90 min $y = 0.0039x^2 - 1.1201x + 100.69$, $R^2 = 0.9911$, 2nd 90 min after spiking, $y = 0.0025x^2 - 1.7305x + 325.27$, $R^2 = 0.9930$; Trend lines and data points for reactions with and without PEG overlapping and difficult to see.

4.8. Reducing Anions and Halides

Real wastewater matrices can contain a plethora of organic compounds and inorganic salts in various concentrations. A variety of reducing anions made from their sodium salts were added in increasing concentration from 0-200 mg/L. Laccase's ability to convert p-cresol, at optimum pH in aerated batch reactors was monitored (**Fig 4.57**).

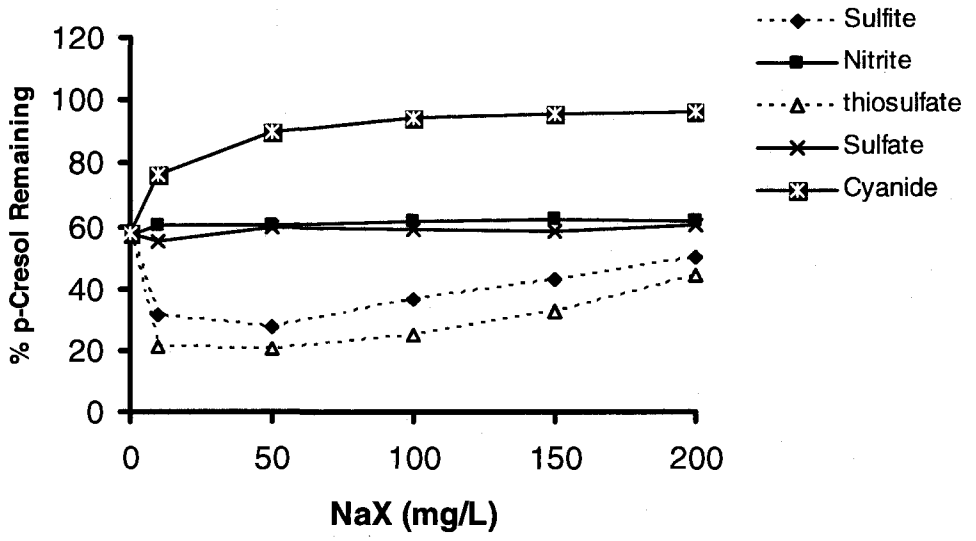


Figure 4.57: Effect of Reducing Anions on Conversion of 1.0 mM p-Cresol. (3 h reaction time; 40 mM phosphate buffer, pH of 5.7; 0.001 LACU/mL, aerated)

A similar experiment was done with a different laccase (*Trametes versicolor*) and BPA in closed batch reactors (Kim and Nicell, 2006). It was observed that some of the inhibition caused by the reducing anions could not be alleviated by aeration suggesting that inactivation is not due to competition for oxidant. This study shows contradictory results in that sulfite and thiosulfate actually showed higher conversion at low concentrations than the control when fully aerated. This was similar to the results observed with HRP and phenol (Wagner and Nicell, 2002; discussed more in wastewater section). With laccase and phenol, elevated removal under stressed conditions was not observed. These

differences can be attributed to the difference in inactivation between phenol (little to none) and p-cresol (~ 88.0%). It was hypothesized that the sulfurous anions may have an affinity for the products generated by the oxidative coupling, acting as sacrificial molecules based on the premise that reduced rates of inactivation were observed (Wagner and Nicell, 2002). The fact that only p-cresol shows increased removal seems to support this theory. Removal over time was monitored and compared to a control to see if a noticeable decrease in initial rates could be observed due to the competition for oxidant with excess enzyme (**Fig 4.58**).

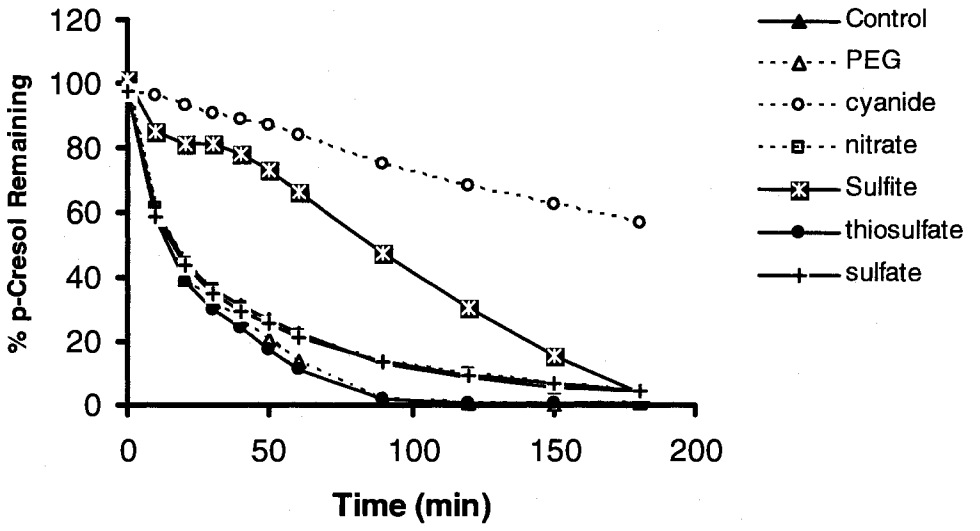


Figure 4.58: Removal Over Time with Reducing Anions for 1.0 mM p-cresol (3 h reaction time; 40 mM phosphate buffer, pH of 5.7; 0.01 – (excess to the optimum done for 3 h LACU/mL; 100 mg/L of the sodium salt, aerated)

From **Fig 4.58** it is seen that sulfite seems to slow the initial conversion, but by the end of the 3 h reaction period removal "catches up". Both PEG and thiosulfate have identical removal profiles, both with higher rates of conversion of p-cresol than the control. Nitrate and sulfate appear to have identical removal rates to the control suggesting they are inert to the system. As expected, cyanide greatly reduced p-cresol conversion although the

mechanism of inactivation is most likely due to its ability to interact with the T2/T3 Cu site competing for the oxygen binding site (Xu 1996) and not through direct competition of oxidant (DO). The fact that thiosulfate has different effects with phenol and p-cresol suggests an interaction with the reaction products as opposed to the enzyme itself (**Section 4.10.5.**). As well as observing the effect of reducing anions, the effect of halides were observed. Both chloride and bromide ions have little or no effect on p-cresol conversion up to 125 mg/L (**Fig 4.59**). Fluoride ions had an increasing inhibition effect with increasing concentration. Inhibition of halide potency varies from laccase to laccase but the order remains the same $Fl^- > Cl^- > Br^-$ which correlates to the accessibility of laccase's T2/T3 Cu site which has a channel with a cut-off diameter excluding the hydrated forms of Cl^- or Br^- (Xu 1996). Halide inhibition is concentration-dependent and is reversible (Xu 1996). Iodide reacts with the T1 Cu site and is considered to be a substrate of the enzyme (Xu 1996). It should be noted that while not tested in this study PEG has shown that it can not protect the laccase against fluoride or cyanide inactivation, nor can it protect peroxidases against temperature and hydrogen peroxide inactivation (Kim and Nicell, 2006a; Wu et al., 1998).

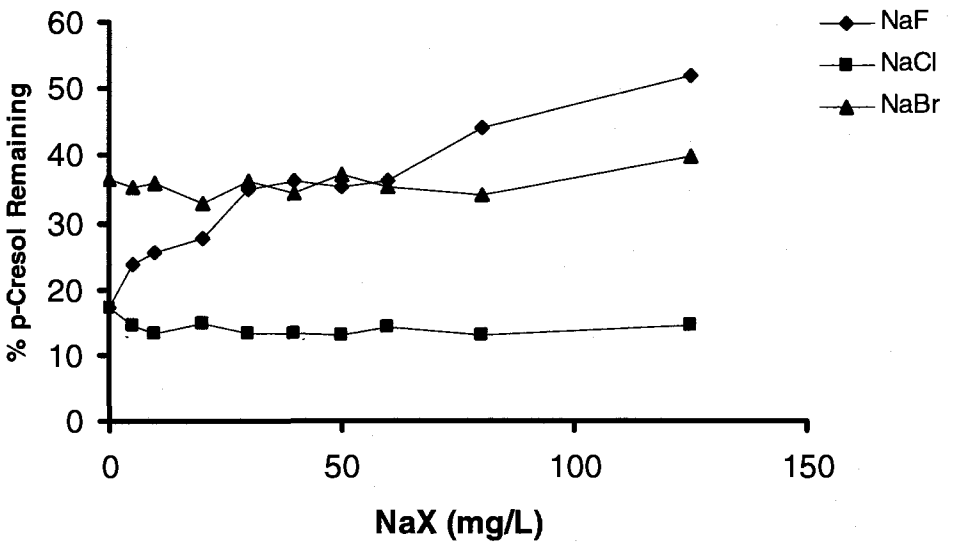


Figure 4.59: Effect of Halides on p-Cresol Conversion (3 h reaction time; 1.0 mM p-cresol; 40 mM phosphate buffer, pH of 5.7; 0.002 and 0.004 LACU/mL for NaBr and (NaCl, NaF) respectivly, aerated)

4.9: Kinetics

When considering reactor parameters, kinetic parameters are usually an asset in giving people a guideline to the general affinity a particular enzyme may have for various substrates. An enzyme with lower affinity that may be cheaper may allow for a more cost-effective treatment regime. In most reactors there will be a series of competing reactions making the analysis quite complex. Initial velocities (**Figures 4.60, 4.61, 4.64, 4.67, 4.68**) were plotted versus the initial substrate concentration in Michaelis-Menten (M-M; **Figures 4.62, 4.65, 4.69**), and Lineweaver-Burk (**4.63, 4.66, 4.70**) formats to obtain apparent kinetic parameters K_M and V_{max} for conditions with and without PEG. Since laccase is a multi-substrate enzyme, we will be focusing on the removal of reducing substrate which has been documented as being the rate determining step in catalysis. It is possible that O_2 may be limiting in this period since extensive O_2 kinetics

were not done so these results would be apparent values based on the reaction conditions it which the experiments were performed. Reactions were formulated with 40 mM phosphate buffer at optimum pH, a slight excess of PEG, and fully aerated. Samples were quenched at 2 min intervals to pH < 2 with H_2SO_4, micro-filtered and analyzed for residual cresol using HPLC. All experiments were run at 22 °C ± 3°C. The increased conversion could be due to two possible mechanisms, the protection of the enzyme, increase in oxidation rate, or a combination of both. It has already been proven that PEG prolongs the enzyme's catalytic lifespan. If the increased conversion is solely due to the extended lifespan of the enzyme then it is expected that the initial velocities, before significant inactivation has occurred and should produce almost identical apparent kinetic parameters. However if there is an increase in oxidation rate in combination with the extended catalytic lifespan of the enzyme we would expect to see varying apparent kinetic parameters.

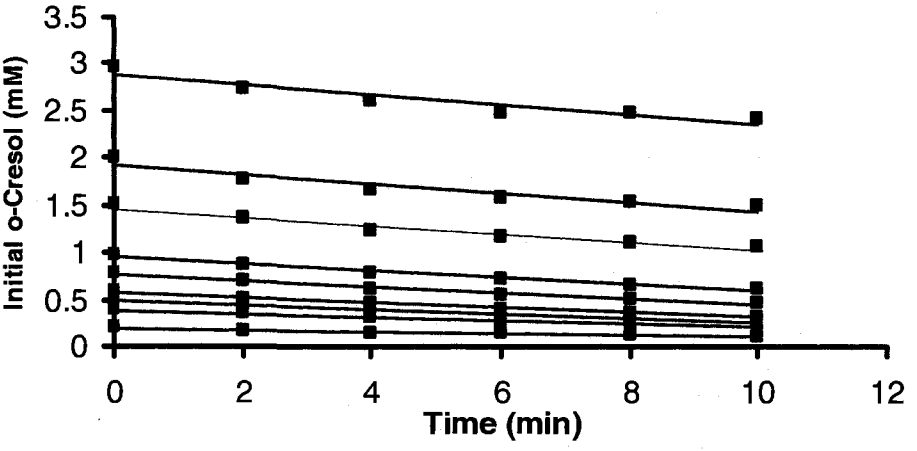


Figure 4.60: Initial Velocities for o-Cresol (without PEG) (reactions with 0.02 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro-filtered and analyzed by HPLC).

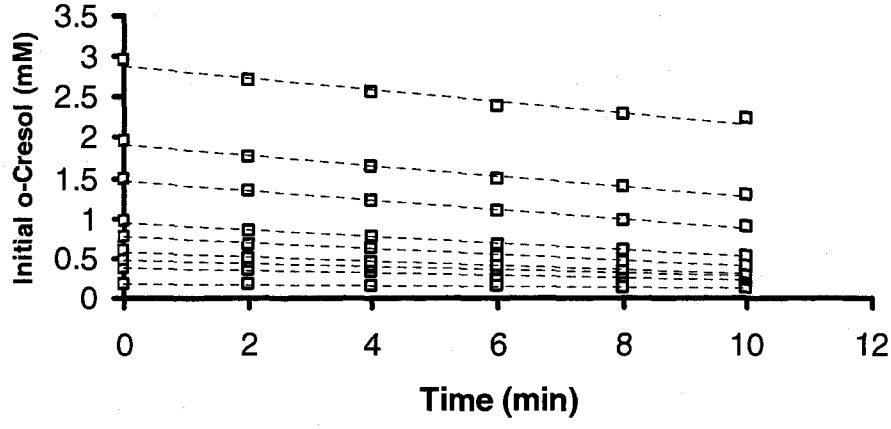


Figure 4.61: Initial Velocities for o-Cresol (with PEG). (reactions with 0.02 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro-filtered and analyzed by HPLC)

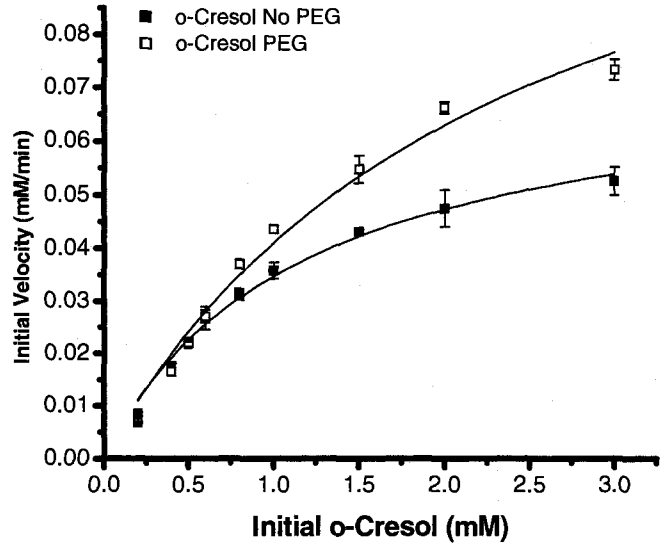


Figure 4.62: Michaelis-Menten Plot for o-Cresol With and Without PEG. Calculated K_M = 1.14 ± 0.11 , V_{max} = 0.074 ± 0.003, R^2 = 0.983, Chi^2/DoF = 9.882 E^{-6} and K_{MPEG} 2.29 ± 0.42 and V_{maxPEG} 0.135 ± 0.014, R^2 = 0.991, Chi^2/DoF = 2.215 E^{-6} (reactions with 0.02 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro filtered and analyzed by HPLC).

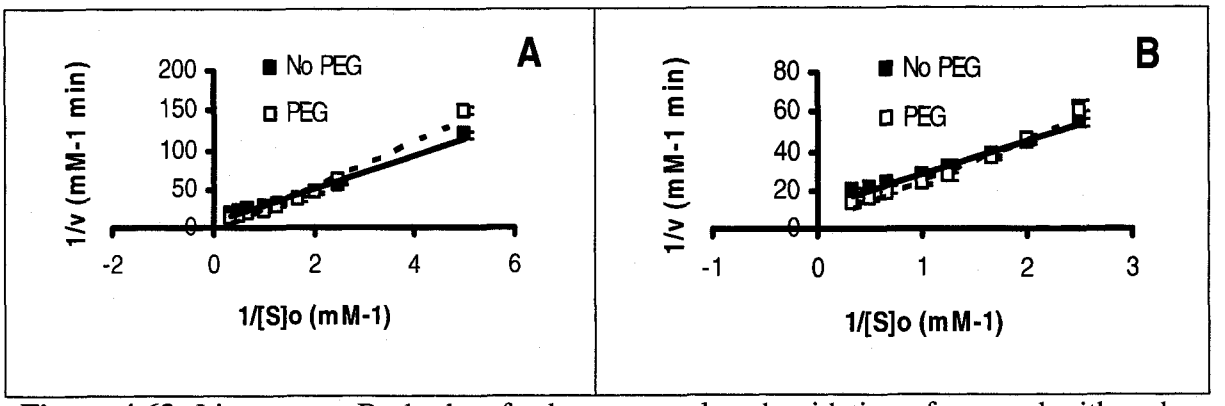
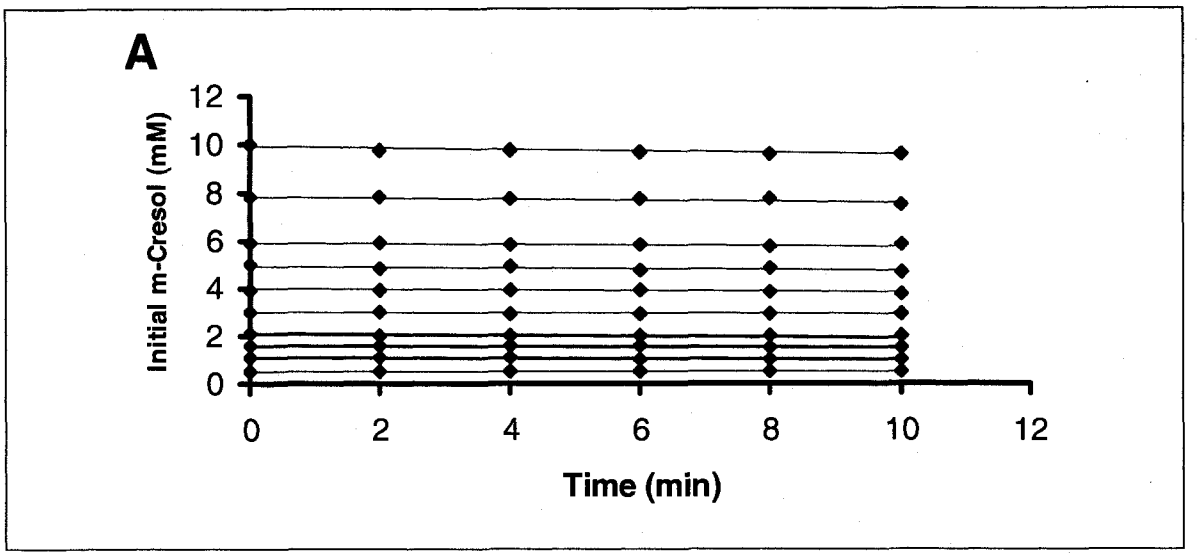


Figure 4.63: Lineweaver-Burk plots for laccase catalyzed oxidation of o-cresol with and without PEG (reactions with 0.02 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro filtered and analyzed by HPLC). **A.** No PEG y = 21.45x + 6.8633, R^2 = 0.983; PEG y = 28.948x – 4.7821, R^2 = 0.9753 **B.** (plotted without lowest $[S]_o$) No PEG y = 16.867x + 11.919, R^2 0.9848; PEG y = 21.374x + 3.5735, R^2 = 0.9783.



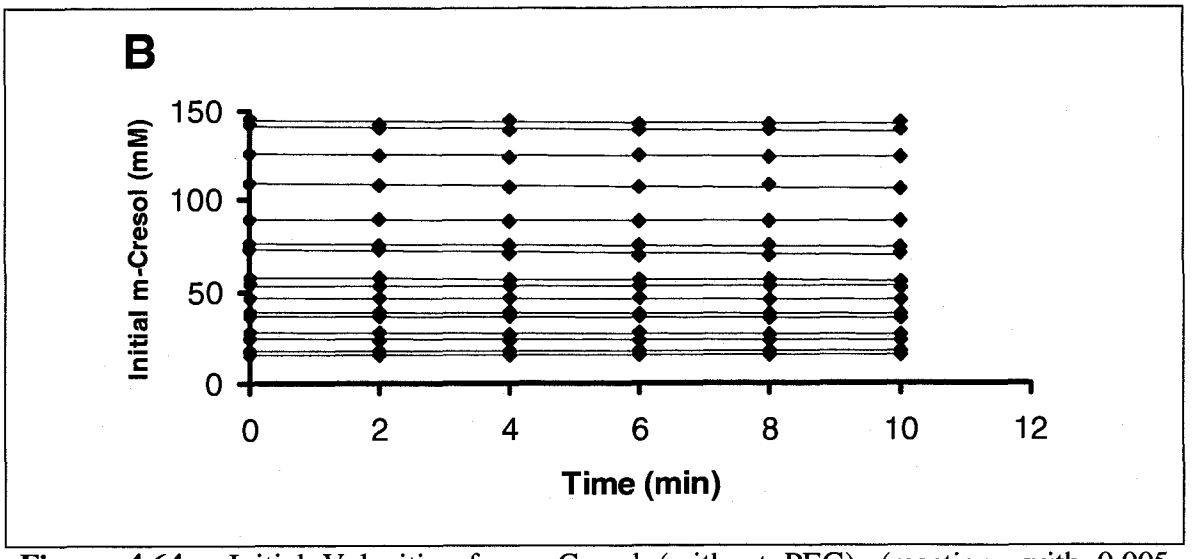


Figure 4.64: Initial Velocities for m-Cresol (without PEG). (reactions with 0.005 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro-filtered and analyzed by HPLC, **(A)** $[S]_0$ 0.25 – 10.0 mM **(B)** $[S]_0$ 14.0 – 145.0 mM)

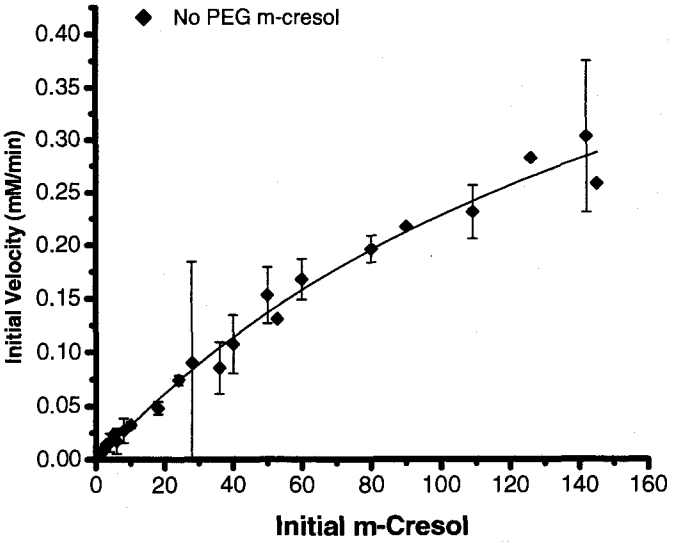


Figure 4.65: Michaelis-Menten Plot for m-Cresol Without PEG. Calculated K_m = 202± 35 mM , V_{max} = 0.688 ± 0.080, R^2 = 0.988, Chi^2/DoF = 0.00012 (reactions with 0.005 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro-filtered and analyzed by HPLC).

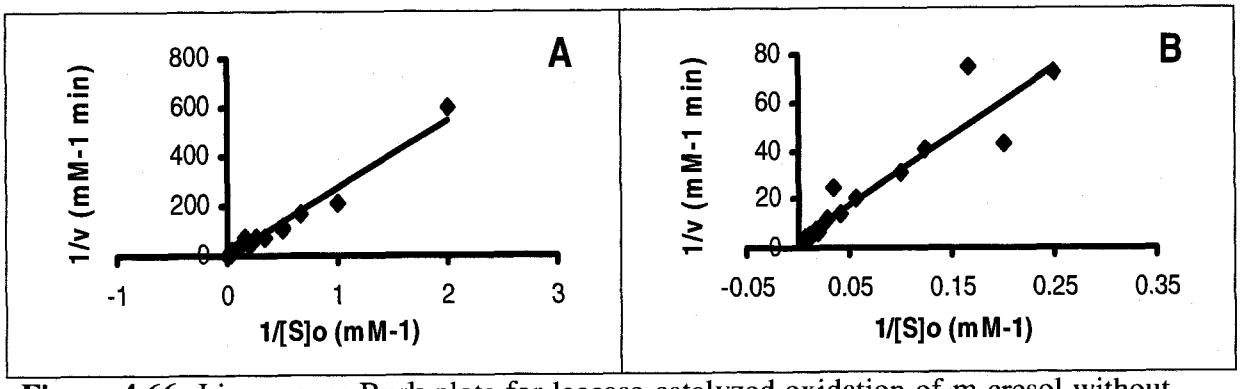


Figure 4.66: Lineweaver-Burk plots for laccase catalyzed oxidation of m-cresol without PEG (reactions with 0.005 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro filtered and analyzed by HPLC). **A.** No PEG y = 275.24x – 1.2426, R^2 = 0.9752 **B.** (plotted from 4.0 mM $[S]_o$ and up) No PEG y = 286.66 + 3.1565, R^2 = 0.8824.

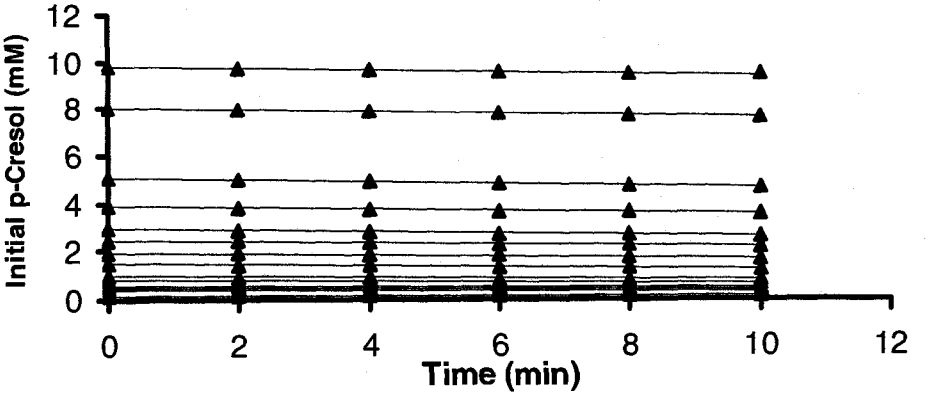


Figure 4.67: Initial Velocities for p-Cresol (without PEG). (reactions with 0.0025 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro-filtered and analyzed by HPLC).

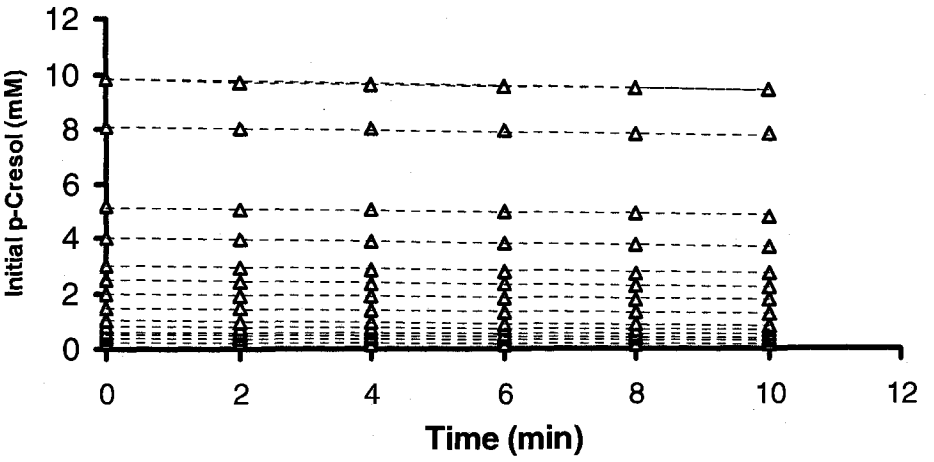


Figure 4.68: Initial Velocities for p-Cresol (with PEG). (reactions with 0.0025 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro-filtered and analyzed by HPLC).

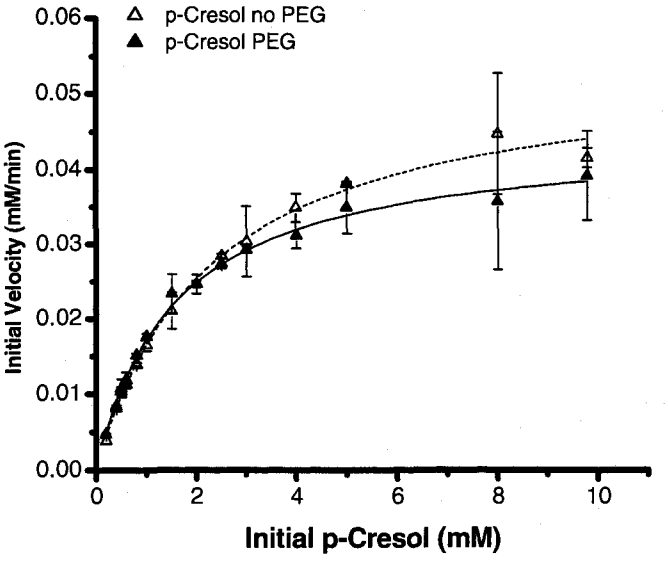


Figure 4.69: Michaelis-Menten Plot for p-Cresol With and Without PEG. Calculated K_M = 2.26 ± 0.15 V_{max} = 0.054 ± 0.002, R^2 = 0.993, Chi^2/DoF = 1.301 E^{-6} and K_{MPEG} 1.58 ± 0.09 and V_{maxPEG} 0.045 ± 0.001, R^2 = 0.994, Chi^2/DoF = 7.675 E^{-6} (reactions with 0.0025 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro-filtered and analyzed by HPLC).

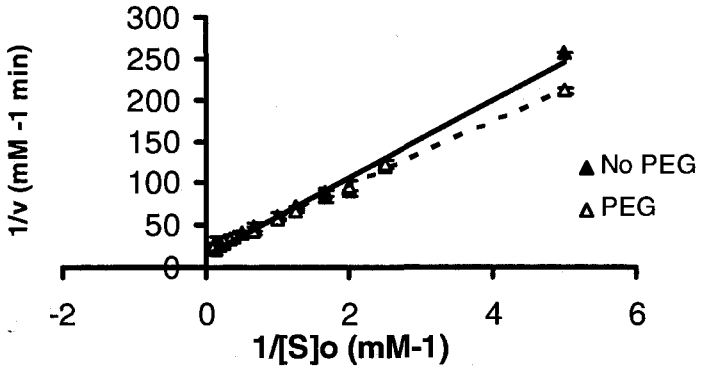


Figure 4.70: Lineweaver-Burk plots for laccase catalyzed oxidation of p-cresol with and without PEG (reactions with 0.0025 LACU/mL; 40 mM phosphate buffer at pH 5.7 quenched with acid < pH 2; micro filtered and analyzed by HPLC). No PEG y = 45.854x + 15.111, R^2 = 0.9891, PEG y = 38.17 + 21.54, R^2 = 0.9973.

Table 4.10: Summary of Kinetic Parameters From Non-Linear Cuve fit of Initial Rate data to the Michaelis-Menten Equation

	o-Cresol (0.02 LACU/mL)		**m-Cresol (0.005 LACU/mL)**	**p-Cresol (0.0025 LACU/mL)**	
	No PEG	**PEG**	**No PEG**	**No PEG**	**PEG**
K_m (mM)	1.144 ± 0.111	2.294 ± 0.042	201.702 ± 34.570	2.258 ± 0.153	1.582 ± 0.088
V_{max} (mM min^{-1})	0.074 ± 0.003	0.135 ± 0.014	0.688 ± 0.080	0.054 ± 0.002	0.045 ± 0.001
V_{max}/K_m (min^{-1})	0.065	0.059	0.003	0.024	0.028
R^2	0.983	0.991	0.988	0.993	0.994

Table 4.11: Summary of Kinetic Parameters Based on Linear Fits of Initial Rate Data with Lineweaver-Burk plots

	o-Cresol (0.02 LACU/mL)				**m-Cresol (0.005 LACU/mL)**		**p-Cresol (0.0025 LACU/mL)**	
	No PEG		**PEG**		**No PEG**		**No PEG**	**PEG**
K_m (mM)	3.125	1.415†	6.05	5.981†	43.73	90.81†	3.034	1.772
V_{max} (mM min^{-1})	0.146	0.084†	.209	0.280†	0.188	0.317†	0.066	0.046
V_{max}/K_m (min^{-1})	0.047	0.059†	0.036	.0468†	0.004	0.004†	0.022	0.026
R^2	0.983	0.985†	0.975	0.978†	0.975	0.882†	0.989	0.997

† plot 4.63,66 B results, with the lower [S] removed

K_M, Michaelis-Menten constant, analogous to the dissociation constant is an indication of the affinity of enzyme for a substrate (lower value means higher affinity, better recognition). By comparing K_M values from the M-M plots (**Table 4.10**) we find that o-cresol has the lowest K_M followed by p-cresol and m-cresol. In the presence of additive, p-cresol's K_M is lowered and o-cresol's is increased. m-Cresol was not done with PEG because increasing additive concentration in relation to the increasing substrate concentration caused what appeared to be the precipitation of PEG since the reactors turned a "milky" white colour. Despite these changes the V_{max}/K_M (catalytic efficiency) ratios are similar under both conditions, within 10% or each other. All of the R^2 values were between 0.983-0.993 indicating a good fit to the non-linear M-M regression. When the same data was plotted in double-reciprocal plots, the linear Lineweaver-Burk plots, had similar trends but larger deviations in apparent K_M and V_{max} values excluding the p-cresol results. In some cases a negative y-intercept indicating a negative V_{max} which is impossible. In order to correct for such error some of the smaller initial substrate concentrations were excluded. An inherent error in using the Lineweaver-Burk occur when taking the reciprocal value of the lower values which enhances errors. Removing a few of the lower substrate values make the kinetic values closer to those predicted by the M-M plots. Even after this subtle manipulation of data o-cresol-PEG K_M and V_{max} are both almost double that of the M-M predicted values. The doubling does not change the V_{max}/K_M value. The R^2 values range from 0.882-0.997. Values for m-cresol vary considerably between the two types of analysis, although it isn't a true comparison because some points are excluded from the Lineweaver-Burk plot. The apparent K_M for m-cresol is drastically different from the other two cresol isomers, more so than was

anticipated at the beginning of this study. It should be noted that the M-M plots for o-cresol should have been taken to a higher concentration so that a more apparent V_{max} could have been observed. A previous study using the same laccase as in this study with BPA with and without PEG found the following apparent kinetic parameters with 0.0011 LACU/mL: with no PEG V_{max} = 0.013 ± 0.001 mM min $^{-1}$, K_M = 0.185 ± 0.043 mM, V_{max}/K_M = 0.07 min $^{-1}$, with PEG V_{max} = 0.047 ± .002 mM min $^{-1}$, K_M = 0.573 ± 0.073 mM, V_{max}/K_M = 0.08 min $^{-1}$ (Modaressi et al. 2007). These results are similar to the o-cresol results in that there is a proportional increase in both V_{max} and K_M with additive. Another study using laccase (*Trametes versicolor*) with and without additive found that the apparent kinetic parameters were not altered by the additive with an apparent K_M of 0.690 mM for BPA (Kim and Nicell, 2006a). These results suggest that the additive is not increasing the rate of oxidation. The results in this study suggest that while catalytic efficiency is not changing (V_{max}/K_M) the rate of oxidation is being altered. When oxygen kinetics was monitored over time with BPA-laccase-PEG it was found to increase the total oxygen uptake when the PEG was present, but the initial velocity (first 2 min) showed no change in oxygen consumption (Modaressi et al., 2007). The oxidation of 1- and 2-napthol in the presence of recombinant laccases, rPpL, rMtL, rCcL and water soluble polymers showed that these polymers could be divided into three groups based on the oxygen consumption. The first group which increased the total oxygen consumption without changing the initial rate, but prolonged the activity of laccase included: PVA, FIC, copolymer of acrylamide, N-vinylpyrrolidone and ethyl acrylate, HEC, D500, D110, D20, PVP and PEG. The second group that inhibited initial oxygen uptake but not total oxygen consumption, thought to interact with the negatively charged laccase since the

polymers were cationic and included: DED, PLL, PEI, protasan. The third group did not affect either the initial or total oxygen consumption and were negatively charged polymers and included: NaA, PAA and HEP thought to have no interaction with enzyme or intermediates (Kulys et al., 2003).

4.10 Refinery Wastewater

All of the previous work has been conducted on synthetic wastewater matrices. However, real wastewater matrices may be much more complex with effluents having fluctuating pH, temperatures, pollutant concentrations and may contain heavy metals, inorganic salts and a plethora of organic compounds (solvents) in various ratios. Thus the purpose of this part of the study was to explore the effectiveness of laccase SP 504 on real refinery wastewater (R) samples. The refinery samples were collected after the sour water strippers but before the biox-reactors and subsequent dilutions. Therefore these grab samples had the highest possible concentration of all contaminants. When the samples were received the initial pH and phenol concentration were recorded (**Table 4.12 page 155**) as well as tested for heavy metals by atomic absorption and cyanide using EM Quant Test strips. A mixed cresol content of up to 0.12 mM was detected in addition to the phenol concentration by HPLC based on comparison with authentic HPLC samples.

4.10.1 pH Optimization

The effect of pH was observed over the range 2.0 – 9.0 under stressed conditions with laccase limiting the reaction to substantially less than full conversion. Reactions were run both with and without PEG. PEG had no effect on conversion so its data was excluded in **Fig 4.71**. 40 mM buffers were added to the refinery samples diluting them

by one-quarter so as to maintain a stable pH (± 0.05) through the course of the 3 h reaction period.

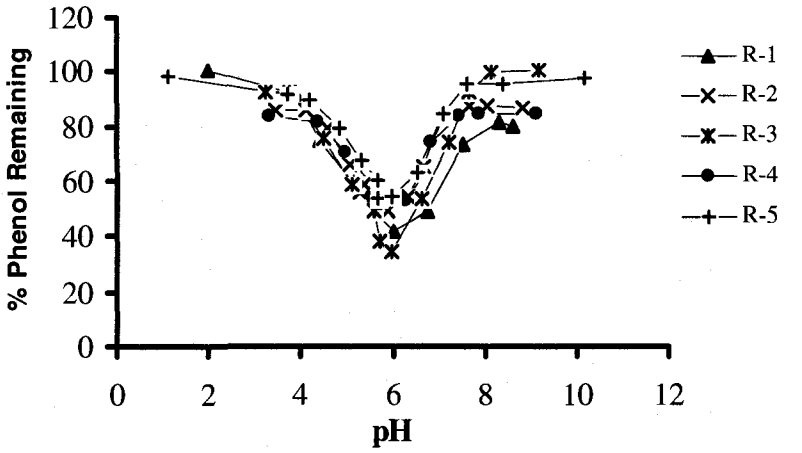


Figure 4.71: pH Optimization for phenol Refinery (R) Wastewater. (40 mM buffers; wastewater one-quarter diluted due to buffer addition; 3 h reaction times; analyzed by HPLC; 0.04, 0.036, 0.038, 0.05, 0.035 LACU/mL for R 1-5 respectively).

Fig. 4.71 displays similar pH optima for all of the refinery samples between pH 5.6-6.0 (**Table 4.12, page 155**). This range is within the expected value for laccase SP 504. The predicted biphasic plot is observed with the increase in conversion due to the reduction in redox potential with increasing pH and the loss of activity due to the increase of hydroxyl radicals competing for the T2/T3 Cu site. Also, little conversion is observed before the conserved Arg(206) (pK_a 3.9) is deprotonated (Tadesse et al., 2008; Madzak et al., 2006; Betrand et al., 2002; Bukh et al., 2006). There is no shift in optimum pH range for the conversion of phenol in the refinery samples, relative to an authentic phenol sample. Since the initial pH of the refinery samples ranged from 6.7-8.7 all of the samples would most likely have to be adjusted in practice for laccase to be useful. It should be noted that pH slowly decreased as the samples aged in the lab. Enzymes that have more robust

pH optima such as SBP may be more applicable in these situations since pH adjustment of effluents can be costly (Steevensz et al., 2008).

4.10.2 Optimum Enzyme Concentration

Refinery samples were pH adjusted with H_2SO_4 to within the predetermined optimum pH range. Increasing enzyme concentrations were added to achieve the desired removal of ≥ 95% with 3 hours' stirring, before being quenched and determination of residual phenol concentration by HPLC. In **Fig 4.72** removal based on enzyme concentration can be observed with percent of phenol remaining based on the initial phenol comcentration which varied to some degree (initial phenol concentration was ~ 1.0 mM except for R-1, **Table 4.12, page 155**).

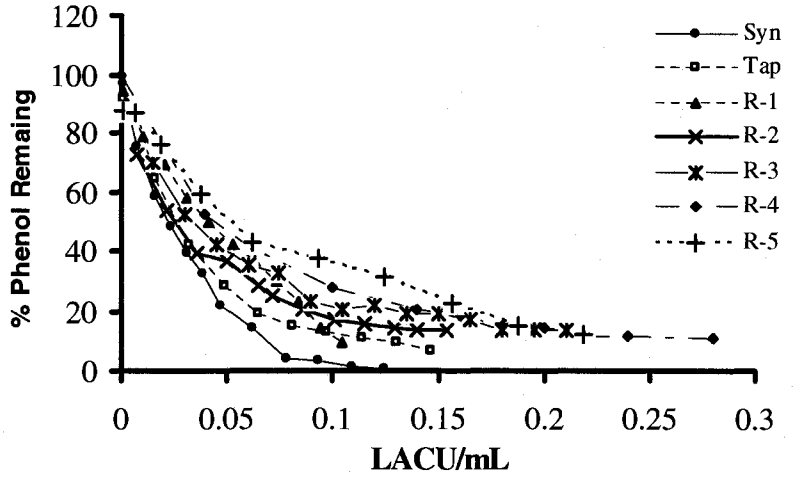


Figure 4.72: Laccase Optimization for phenol Refinery Wastewater. (40 mM 5.8-6.0 phosphate buffers used; R-1-5 initial phenol in **Table 4.12;** for Syn, 1 mM phenol and tap waster adjusted with dilute NaOH; 3 h reaction time; Syn- Synthetic, R- Refinery Samples)

1. Reactors containing 100 mg/L of PEG run in parallel to the samples in **Fig 4.72** showed no changes, similar to the results with synthetic phenol. Removal efficiencies were greatly reduced for the refinery samples with conversion

leveling off at 90 - 95% despite the addition of 1.5-2.8 times more enzyme. The small amount of cresols (0.12 mM) should not account for such a large increase in enzyme concentration required since they have been shown to be better substrates of the enzyme (**Section 4.2**). Why conversion levels off is unknown because in theory incomplete conversion should be overcome by increasing the enzyme concentration. It could be speculated that substances in the matrix are adsorbing some of the free enzyme, affecting its stability, forming reversible inhibitor complex with the enzyme, or oxygen diffusion is inhibited, none of which can be confirmed without further investigation.

4.10.3 PEG effect on Refinery Wastewater

To ensure PEG had no effect, as observed in the pH study and synthetic sample the refinery samples were run with increasing concentrations of PEG (0-400 mg/L) that had been pH-adjusted using H_2SO_4, run under stringent conditions where laccase was limiting to substantial conversion.

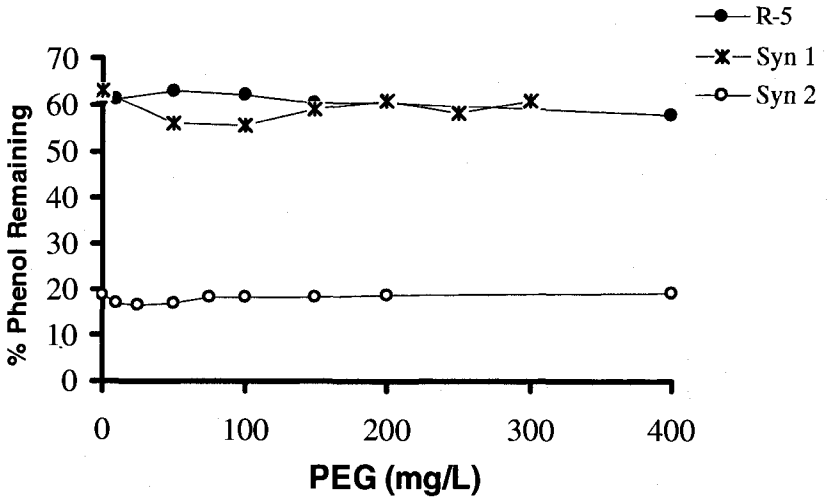


Figure 4.73: Effect of PEG on Synthetic and Refinery Wastewater. (3 h reaction; PEG_{3350}; 40 mM 5.7 phosphate buffer for Syn; pH adjusted with H_2SO_4 for R-5; 1.0 mM Phenol (Syn 1 and 2); 0.015 LACU/mL for Syn 1, 0.05 LACU/mL for Syn 2 and R-5; Syn- Synthetic, R- Refinery Samples)

Fig 4.73 shows R-5 as a representative of all the refinery samples which, like the synthetic samples, is unaffected by the addition of PEG. From this study it was speculated that laccase in the refinery samples is not being inactivated by the polymeric products.

4.10.4 Removal and Activity Over Time of Refinery Samples

Since enzyme requirements of the refinery samples were 1.5-2.8 times greater than the synthetic samples the immediate assumption was that something is inhibiting phenol conversion through the inactivation of laccase. Since PEG had no effect on reducing the amount of enzyme it was thought that inactivation due to polymeric products was not the primary source of increased enzyme demand. Therefore activity over the three hours as well as conversion was monitored (**Fig. 4.74**).

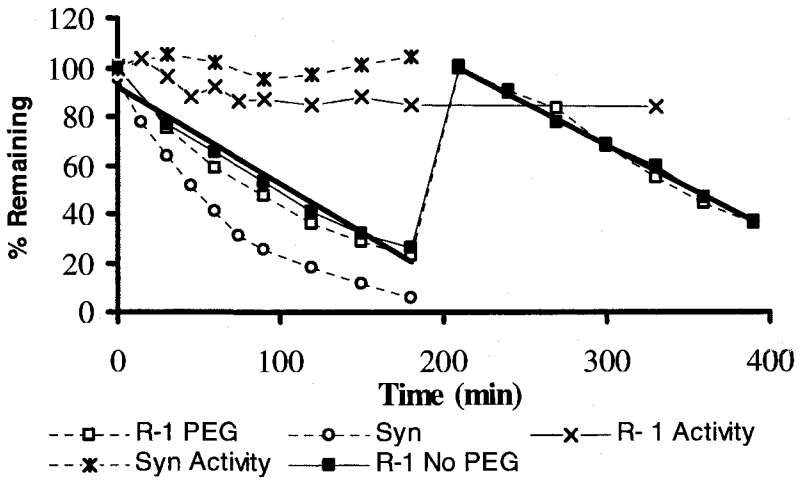


Figure 4.74: Activity and Removal Over Time of Synthetic and Refinery Wastewater. (40 mM phosphate buffer pH 5.8, R-1 Spiked after 3 h back to initial phenol concentration; both synthetic and R-1 0.08 LACU/mL; Syn- Synthetic, R- Refinery Samples) Linear equation for 1st 3 h: $y = -0.4015x + 92.484$, $R^2 = 0.9687$, for 2nd 3h (after spiking): $y = -0.3511x + 173.85$, $R^2 = 0.9984$.

During the first three hours little activity was lost in refinery and synthetic samples. Since the refinery sample had many uncharacterized components and it is possible that the activity assay may be compromised due to lack of a valid control, removal was monitored for the first three hours, then spiked back to ~ 1.0 mM and again the removal monitored. The removal of the synthetic samples occurred quicker than the refinery samples. However the removal rates between the first 3 h and the second 3 h in the refinery sample only showed a decrease in removal rates by 14.0% when comparing the two slopes (**Fig 4.74**). The decrease in removal correlates closely to the average loss in activity in the synthetic samples. **Fig 4.74** also exemplifies how slow laccase is at converting phenol. When the pH of the reactors was not adjusted, removal was even slower and even after 36 h (data not shown) significant phenol conversion was not observed. Therefore it is imperative that pH adjustments be made if removal of substrate is to occur at a reasonable rate with laccase SP 504.

4.10.5 Reducing Anions and Halides

From the previous results it seems as though enzyme inactivation is not the source of the increased enzyme demands and reduced rate of phenol conversion. It was then hypothesized that contaminants in the wastewater matrix may be competing for oxidant. Similar studies on the same refinery samples were being run with SBP and from these studies it was observed that there was a much larger H_2O_2:SBP requirement than for the synthetic samples (Mousa 2008). Some of the refinery samples consumed up to 30 mM H_2O_2 compared to the control which consumed 5-10% of the optimum concentration in the first 3 h. When the refinery waste was pretreated with aliquots of 10 mM H_2O_2 until the rate of consumption was greatly reduced, followed by enzymatic treatment, both H_2O_2 and SBP were reduced to the amounts required by the synthetic samples (Steevensz et al., 2008). Since laccase does not use H_2O_2 but O_2 as oxidant an attempt was made to monitor oxygen consumption over time but reliable reproducible results could not be obtained due to the dissolved (DO) probe membranes' being clogged by pre-existing solids and oils in the wastewater matrix. Bubbling O_2 through the samples over night did not noticeably reduce the laccase requirement for phenol removal. Studies done in the presence of ions commonly found in petrochemical effluents were carried out to assess the effect they may have on laccase when present at 200 mg/L made from the following sodium salts: sodium sulfite (Na_2SO_3), sodium sulphate (Na_2SO_4) sodium nitrate ($NaNO_3$), sodium thiosulfate (NaS_2O_3), sodium cyanide (NaCN). These reactors were aerated and run at optimum pH, for 3 h with laccase being the limiting factor to complete conversion.

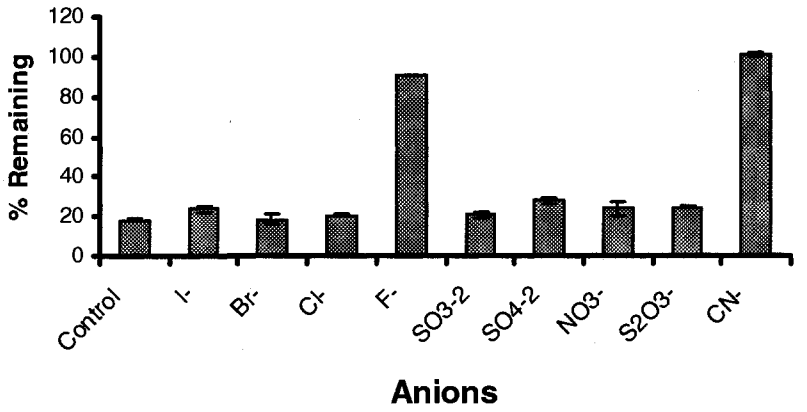


Figure 4.75: Effect of Reducing Agents on Phenol Conversion in Synthetic Wastewater.(3 h reaction; 40 mM 5.8 phosphate buffer; 200 mg/L for reducing anions; 0.05 LACU/mL)

None of the reducing anions showed a significant effect on phenol conversion except for cyanide. Previous studies with HRP and phenol and BPA with *Trametes versicolor* (Lc.) highlighted how some of these ions can have a negative effect on substrate conversion by competing with the enzyme for oxidant, under oxidant limiting conditions (Kim and Nicell 2006; Wagner and Nicell, 2002). In these cases the high levels of phenol conversion could still be attained by aeration with laccases or by increasing the H_2O_2: substrate ratio with peroxidases. With HRP and phenol both sulfite and thiosulfate actually increased the phenol transformation rate when H_2O_2 was not limiting. It was hypothesized that the sulfurous anions may have an affinity for the products generated by the oxidative coupling, acting as sacrificial molecules based on the premise that reduced rates of inactivation were observed (Wagner and Nicell, 2002). This was similar to the p-cresol results in **Section 4.8**. With SBP when no enzyme was added to the batch reactors the 200 mg/L sodium thiosulfate and sodium sulfite consumed 80% and 54% of the initial hydrogen peroxide, but since 1.0 mM phenol could still be removed by adding excess H_2O_2 it was concluded that the addition of excess oxidant can overcome the direct

competition of the reducing anions as observed with HRP (Steevensz et al., 2008; Mousa 2008; Wagner and Nicell, 2002). Studies with *Trametes versicolor* laccase and BPA showed similar results with the exception that thiosulfate almost completely inactivated the enzyme and its effects were not reversed by aeration (Kim and Nicell, 2006). The fact that aeration could not reverse these effects suggests another type of inactivation other than direct competition for oxidant. When thiosulfate was used with laccase SP 504 the colour changed from brownish (424 nm) to reddish (474 nm) with little or no precipitate formed during phenol transformation. The colour change implies an alternative set of products, but since laccase activity loss is very low an increase in removal efficiency is not anticipated as was observed with p-cresol (**Section 4.8**). Cyanide completely inactivated laccase, thought to compete for the T2/T3 Cu site for oxygen binding (Xu 1996). Cyanide also inhibits peroxidases, thought to diffuse through the distal cavity and alter the co-ordination number of iron from 5 to 6 (Dunford, 1999). Since cyanide is used in numerous condensation and polymerization reactions in the plastics industry and it is often released in petroleum and petrochemical effluents some sort of pretreatment would be necessary before the enzymatic process, if it were present. The refinery wastewater tested negative for cyanide by EM Quant Test strips. The effect of halides, fluoride, chloride, bromide and iodide, as their sodium salts (200 mg/L) was investigated. Reactors were run under stressed conditions for 3 h, at optimum pH, with laccase being the limiting factor to phenol conversion. It was found that only fluoride ions severely impaired the conversion of phenol (**Fig 4.75**). Laccases have variable tolerance towards individual halides but all appear to have the same order of inhibitory potency as follows: $F^- > Cl^- > Br^-$ (Xu 1996). The order of inhibitory potency has been

attributed to the accessibility of laccase's T2/T3 Cu site which has a channel with a cut-off diameter of the hydrated forms of Cl^- or Br^- (Xu 1996). The inhibitor effect has been found to be reversible since full recovery of laccase was observed when the halide concentration was reduced to 100 fold its I_{50} (concentration for 50% activity loss). Iodide reacts with the T1 site and is therefore considered as a substrate of laccase, in this study it reduced phenol conversion by 5 - 10% (Xu 1996).

4.10.6 Heavy Metals

It still had not been determined why the refinery sample had such high enzyme demands. Previous studies had demonstrated that laccases were relatively insensitive to metal ions besides Fe^{+2} (Hatvani and Mecs, 2003). It has also been reported that Cu^{2+} in the range 0.1 – 1.0 mM had positive effects on laccase activity; at greater concentrations reduced substrate conversion was observed (Lorenzo et al., 2005). Therefore, the refinery samples were tested for Fe^{3+}, Pb^{2+}, Cu^{2+}, Cd^{2+} Mg^{2+}, and Zn^{2+} by atomic absorption. All the metals analyzed for were found at concentrations similar to tap water, below 0.5 mg/L. These results suggest the higher enzyme demands are not due to metal ions.

4.10.7. Colour Removal

After the enzymatic treatment of phenol, the remaining aqueous solution had a brownish colour with a λ_{max} in the visible range at 424 nm. Laccase products did not settle even after a 24 h settling period. In an attempt to remove the coloured products from the reactors aluminum sulfate (alum) was added in increasing concentration from 0.0-2.5 mM followed by a pH adjustment to 7.0-10.0 to ensure gel was formed and allowed to settle for 3 h (**Fig 4.76**).

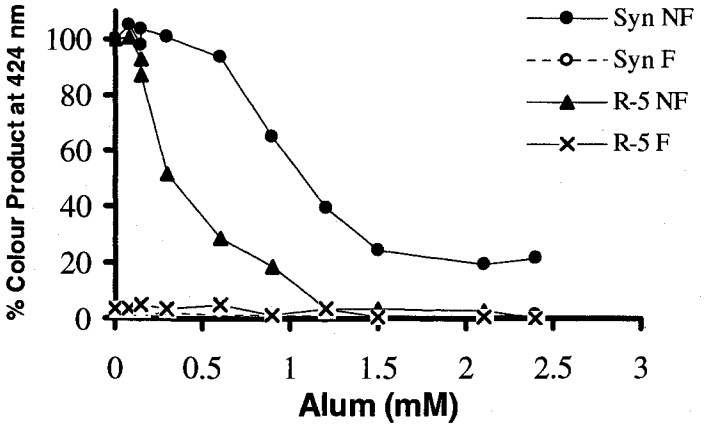


Figure 4.76: Colour Removal Using Alum on Synthetic and Refinery Wastewater. (3 h reactions; 40 mM pH 5.7 phosphate buffer for synthetic, pH adjusted to 5.7 using H_2SO_4 for refinery samples, pH adjusted between 7-10 after alum addition, 3 h settling time)

Even with no alum, micro-filtration removed ≥ 95.0% of the coloured products. Refinery sample R-5 required 1.25 mM alum to settle ≥ 95.0% of its coloured products while with the synthetic samples only 77.0% removal was obtained even with 2.25 mM alum. Why the refinery samples had higher colour removal is not known but it can be speculated that that some of the contaminants may be involved in some sort of co-polymerization process. The final results for the refinery samples are summarized in **Table 4.12**.

Table 4.12: Summary of Refinery Wastewater Samples

Sample	Initial pH	Initial Phenol (mM)	Optimum Nominal [E] LACU/mL	Optimum pH Range	Rxn Time (min)	Loss of Activity (%)
Synthetic	N.A	1.0	0.08	5.6-6.2	180	5-20
Tap	6.2-6.8	1.0	0.12	N.D.	180	N.D.
R-1	6.8	0.86	0.09	5.6-6.0	180	8
R-2	7.8-8.1	1.03	0.12*	5.7-5.9	180	15
R-3	7.7	1.0	0.18*	5.7-6.0	180	5
R-4	8.0-8.7	0.95	0.22*	5.7-6.2	180	10
R-5	7.3	0.96	0.18*	5.6-6.0	180	15

N.D. – Not Done, * Removal to 90%.

4.11 Comparison of SBP to Laccase

Experiments were conducted using SBP on the same refinery samples. A comparison was made between Laccase SP 504 and SBP as was alluded to in the preceding sections (Steevensz et al., 2008).

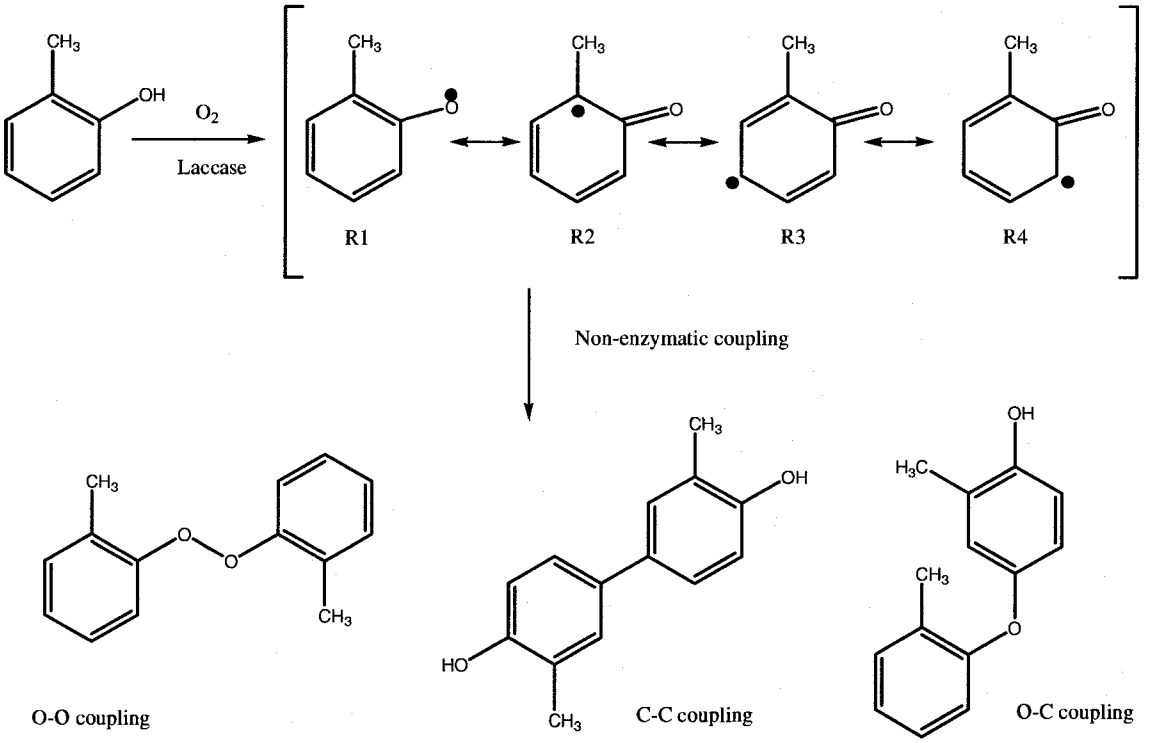
1. Laccase has a narrow optimum pH (5.6-6.0) in which case sample pH adjustment would be required. SBP had a much broader pH range (6.0 – 9.0), hence samples did not require pH adjustment.
2. Using the predetermined minimum enzyme required for ≥ 90 % removal it was observed that laccase was slower at removing phenol but retained its activity, whereas SBP removes phenol quickly (30-90 min) and is inactivated more quickly. Thus the SBP really did not require the entire 3 h reaction period.
3. Halides had no effect on SBP except for fluoride, which almost completely halted the conversion of phenol.
4. Both enzymes were inactivated by cyanide. Thus some sort of pretreatment would be required if any cyanide were present in the effluent.
5. Heavy metals were not present but may be problematic depending on the concentration levels.

4.12 Preliminary Product Identification

Products made from enzymatic oxidation using peroxidases and laccases have not been extensively investigated. One of the better examples showed a proposed coupling mechanism for BPA however it should be noted that the solid precipitate extracted was not completely redissolved in organic solvent and only the compounds soluble in dichloromethane were analyzed (Huang and Weber, 2005). Little work to characterize

the toxicity and mobility of these compounds have been done but it can be speculated that these products are less toxic than the initial substrate and would be safe enough to be disposed of in landfills due to the fact they are highly insoluble in any solvent, aqueous or organic. To complicate issues, even less work has been done on the characterization and comparison of products made in the presence and absence of additives such as PEG. As previously stated these same enzymes have received much attention in recent years for the synthesis of phenolic resins as a green alternative that utilizes a simple procedure and is run under mild conditions compared to the use of formaldehyde, which is highly toxic, as monomer for conventional synthesis of phenolic resins (novolac, resol resins) (Kim et al., 2004; Mita et al., 2003; Kim et al, 2003; Oguchi et al., 2002). It has been demonstrated that coupling selectivity can be altered by changing the hydrophobicity of monomer and solvent in aqueous organic solvent (Mita et al., 2003). When using laccase *Pycnoporus coccineus* in aqueous organic solvent (acetate buffer (pH 5.0) – water, 1:1 ratio) an average phenylene/oxyphenylene ratio 48:52 was found in the product mixture. Products also had an almost identical FTIR spectrum to products generated by HRP (Mita et al., 2003). The MWs of these products are much higher than anticipated in just water or buffered solution. In our study we realize that isolating and generating specific compounds from the mixture generated would be tedious and time intensive. The radical coupling mechanism would be hard to control and thus a large range of products would be expected. Symmetrical compounds such as BPA, phenol, syringic acid, p-cresol etc... would be easier to predict because of the reduced number of different resonance structures (coupling sites) for the reactive intermediates. **Scheme 4.1** illustrates some of the possible dimer products for o-cresol from O-O, C-C, C-O, coupling. Since these

dimers are often better substrates of the enzyme they too can react and couple with monomers, other dimers, or other oligomers resulting in a large array of products. Even if PEG did not protect the enzyme but still affected the regioselectivity of the coupling so that more uniform products were made and thus more likely to be recycled, may still be beneficial. Therefore preliminary product identification has been done to characterize and identify the differences between products made in the presence and absence of PEG_{3350}.



Scheme 4.1: Some of the Possible Dimer Products for o-Cresol.

Prior experiments revealed that there may be PEG or products that contribute similar proton NMR peaks so dialysis of the enzyme was run over four days prior to running the batch reactors. TOC confirmed the removal of organic carbon in each of the washes and the enzyme retained 74 % of its initial activity as compared to a control. For product analysis 10.0 mM o-cresol batch reactors of 50 or 500 mL were run at optimum

conditions (40 mM phosphate buffer) with excess enzyme and excess PEG when applicable to least 95 % completion. The mixture was centrifuged at 8000 rpm for 10 - 15 min and then filtered. The filtrate was analyzed by TOC and UV. Both reaction solutions had a UV absorbance 404-410 nm with a yellowish green colour. Even though residual o-cresol was determined by HPLC the presence of soluble products makes it impossible to quantify the PEG content. The samples were dried and then resuspended in water and spun for 15 min at 8000 rpm. Each of these washes were also analyzed by TOC and UV. Each wash has small aromatic peaks at 270 and 404-410 nm suggesting some soluble product washing off (very little) (**Table 4.16**). The first wash does have a significant TOC value but the UV at 270 would indicate a cresol concentration below 0.1 mM again confirming soluble product or perhaps enzyme being washed away. The products were dried in a dessicator over the weekend with P_2O_5, then dissolved in DMSO-d_6 overnight and analyzed by ^{1}H-NMR. The NMR spectrum confirmed that PEG was present with the polymeric products (**Figure 4.78**).

Table 4.13: TOC and UV of o-cresol product washes

	No PEG			PEG			
	Avg TOC	Abs at 270 nm	Abs at 408 nm	Avg TOC	Abs at 270 nm	Abs at 408 nm	Volume of Wash (mL)
Wash 1	41.01	0.1108	0.1621	34.607	0.0393	0.1368	25.0
Wash 2	5.069	0.0299	0.088	20.258	0.011	0.0777	25.0
Wash 3	2.464	0.0222	0.062	3.450	0.0083	0.0426	25.0
Final Filtrate	2.434	0.0142	0.044	1.977	0.0175	0.0268	25.0

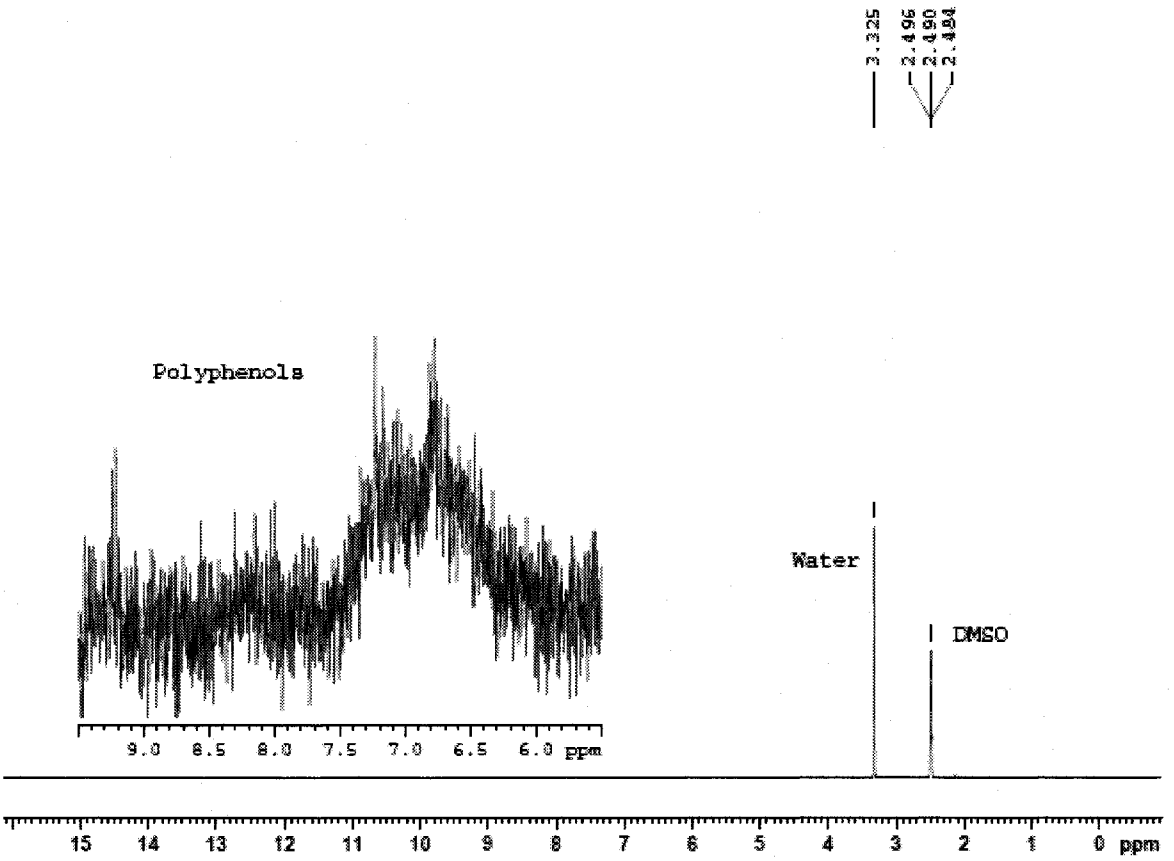


Figure 4.77: ^{1}H-NMR of o-Cresol polymeric products made without PEG. PEG – CH_2 δ 3.49, H_2O δ 3.3. Ar 6.2-7.7 Ar-OH 9.2-9.7.

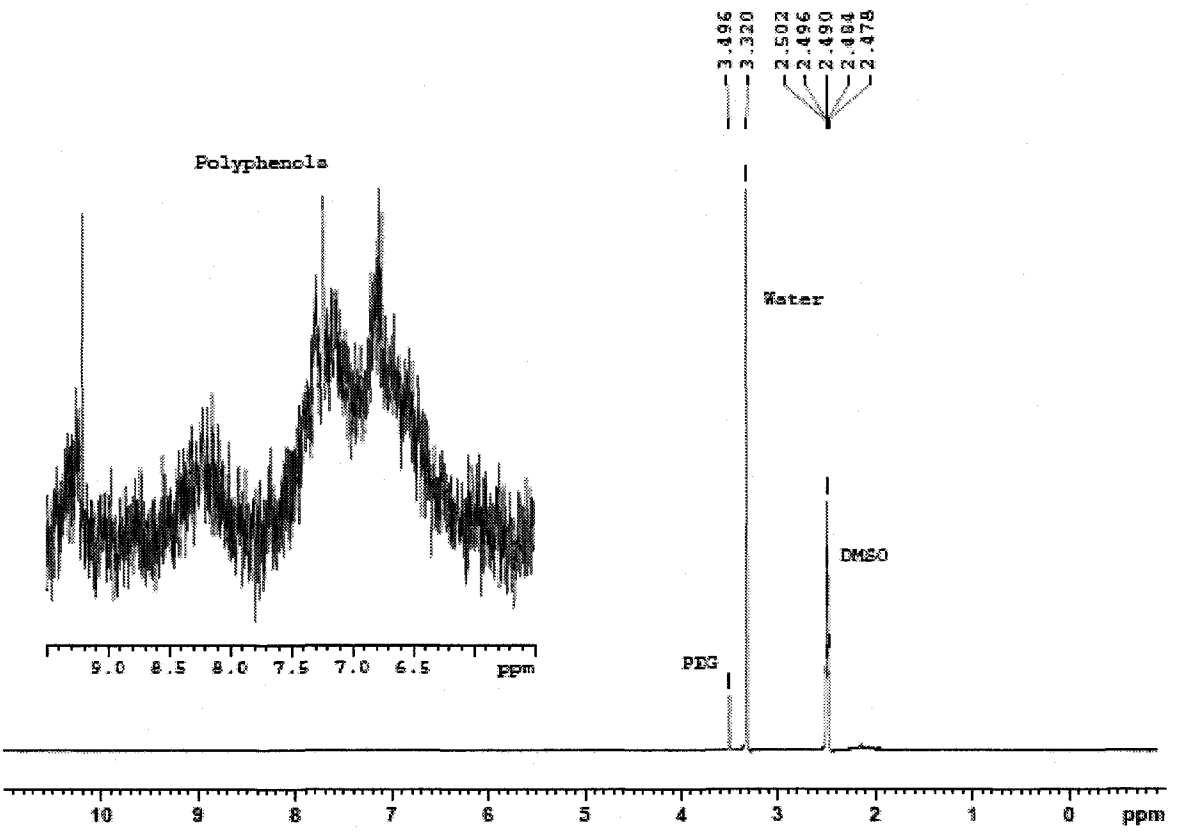


Figure 4.78: ^{1}H-NMR of o-Cresol polymeric products made with PEG. PEG – CH_2 δ 3.49, H_2O δ 3.3. Ar δ 6.2-7.7 Ar-OH δ 9.2-9.7.

PEG appeared at δ 3.49 ppm, water at δ 3.3 ppm, and a solvent peak ~ δ 2.5 ppm which correlates well with expected literature values (Gottlieb et al., 1997). The polyphenol product is expected to be a mixture of compounds with various branching and MW. Exact structures would require further separation and have to be concentrated to determine exact structures. Also if the product was formed via a zip or pick-up mechanism additive concentration and reaction conditions may also play a role in product distribution.

Chapter 5: Summary of Results

Based on batch experiments with and without PEG as an additive, the following summary is made about laccase SP 504 and the conversion of various phenols and anilines:

5.1 Of the eight substrates examined in this study all had similar optimum pH ranges, between 4.7 and 6.6 despite the large differences in their pKa's (4.63 aniline - 10.2 o-cresol) (noting that pKa for amines refer to their conjugate acid). These results are not surprising and followed the bell shaped plots based on the predicted effects attributed to the opposing effect of redox potential at the T1 Cu site (decrease with increasing pH) and the binding of hydroxide anions to the T2/T3 Cu tri-cluster at higher pH. The addition of PEG decreased sensitivity in the acidic pH range for o,p-cresols.

5.2 Minimum enzyme concentrations required for $\geq$ 95 % removal of initial substrate over a range of 0.25 - 2.5 mM showed a linear trend, with and without PEG. PEG significantly reduced the minimum enzyme concentrations required for the cresols and when taking a ratio of the slopes generated by plotting minimum enzyme for $\geq$ 95 % removal versus initial substrate concentration the PEG effects for o-, m-, and p-cresols were found to be 27.9, 2.5 and 11.7, respectively. Phenol and the aniline compounds had no PEG effects. Even with 5 – 10 times more enzyme the amino compounds seemed to level off with 10-20% of the initial substrate remaining. It is not known why this occurs, yet if these reactors were allowed to stand for 24 h $\geq$ 95% removal for m- and p-toluidines could be achieved and $\geq$90% for aniline and o-toluidine. Laccase was much more effective in converting the phenols.

5.3 Minimum PEG concentration for the cresols had a linear trend from 0.5-5.0 mM of the cresols. Since there is a distinct linear trend it can be speculated that there is a

stoichiometric ratio between the polymeric products or reactive intermediates that inactivate laccase and PEG. o-, m-, and p-Cresol slopes indicate that they required ~ 2.6, 27, 3.8 mg/L of PEG per mM of substrate, respectively. Why the m-cresol requires so much more PEG is not known. These PEG concentrations are lower than what has already been determined for the peroxidases. No PEG effect was found with any of the aniline compounds or phenol.

5.4 Varying the MW of PEG and determining its optimum PEG concentration demonstrated that PEG_{400} is ineffective as an additive while MW of ≥1450 all had similar results. The higher MW PEGs required slightly higher concentrations (additional 5-10 mg/L) to achieve the optimum PEG effect. PEG with MW ≥ 1450 had increased substrate conversion following the 3 h reaction period when reactors were allowed to sit for 24 h suggesting the enzyme has not been fully inactivated as compared to the control in which removal seems to level off, indicative of the enzyme being inactivated leaving a large amount of unconverted substrate.

5.5 It was found that only slightly more than the minimum PEG concentration is settled out of solution with the solid product. All values were within 10.0 mg/L of the predicted value except 3.0 mM p-cresol which had almost 4 times the predicted minimum PEG concentration, however due to the experimental set up this could be overestimated. For concentrations above this threshold, PEG accumulates linearly in the supernatant as can be determined from TOC measurements.

5.6 After suspending the products in water and centrifuging the mixture, the supernatant was tested for residual TOC to see if any of the PEG was washed off. No TOC was detected suggesting that the PEG is strongly associated with the polymeric products.

5.7 Laccase activity over time was monitored in batch reactors and it was found that for all of the compounds that a PEG effect was observed with, significant inactivation occurred (38-88%) within the 3 h reaction period. PEG_{400} had no protective effect. Phenol retained almost 100% with an average loss of 10-14%. m-Cresol with the smallest PEG effect had the slowest rate of inactivation. PEG was capable of substantially retaining enzyme activity with losses ranging from 2-12% comparable to the control for the 3 h reaction period. The aniline compounds retained activity after the first 3 h reaction period.

5.8 Laccase SP 805 had previously been determined to have no PEG effect with cresols. The activity over time for the enzyme was monitored and it was observed that there was very little activity loss over the 3 h reaction period. These results are consistent with the sacrificial polymer theory, in that when the enzyme is not inactivated, PEG does not improve conversion efficiency. Laccase SP 504 is more reactive but subject to inactivation more readily. While the source of laccase SP 805 is not known it seems to have characteristics more towards a plant source than a fungal source.

5.9 Monitoring the removal of substrate over time parallels the activity over time results in that when activity is reduced conversion of substrate levels off under stressed conditions. With o- and p-cresols, the majority of the substrate conversion must be completed in the first 100 min after which the inactivation becomes too great for complete conversion. However in the presence of PEG, conversion continues through the 3 h reaction period, higher substrate conversion can be obtained by simply prolonging the reaction time when using lower enzyme concentrations.

5.10 Reducing anions had little effect on p-cresol conversion. Thiosulfate and sulfite had slightly higher conversions then the control reaction when aerated and it can only be speculated that sulfurous anions may have an affinity for the products generated by the oxidative coupling, acting as sacrificial molecules. Both had no effect on phenol removal in which the enzyme is not inactivated. Cyanide can completely inactivate the enzyme. In the presence of halides made from the sodium salt F^- ions reduced p-cresol and phenol conversion and became more potent with increasing concentration, while Cl^- and Br^- had little or no effect.

5.11 Kinetics showed that similar enzyme specificity constants (V_{max}/K_M) were obtained with and without PEG. This was due to proportional increases in V_{max} and K_M. Studies in the past have found similar results, as well as results that show initial velocities are identical which is what would be expected if the enzyme was not inactivated.

5.12 Laccase was only capable of converting 80% of phenol in refinery wastewater samples and required 1.5-2.8 times more enzyme than synthetic samples. A similar pH optimum was observed for all 5 samples, between 5.6-6.0. PEG did not increase conversion efficiency even at 400 mg/L. Activity was retained over the 3 h reaction period and when comparing removal rates for the initial 3 h reaction and a second 3 h reaction period after spiking the sample back to ~100% of the initial phenol concentration there was only a 14% decrease in conversion rate. Since there was little inactivation, no cyanide was detected, and no heavy metals were detected in concentrations above tap water. No conclusive statement can be made as to why the refinery samples required 1.5-2.8 times more enzyme.

Chapter 6: Conclusions

Laccase SP 504, from *T. villosa,* a fungal laccase, was much more proficient in converting the phenol substrates than the aniline compounds which required 5-10-fold more enzyme without achieving complete conversion. Consistent with most fungal laccases, SP 504 had a global pH optimum of ~ 5.6. It was also found that like the peroxidases, a "PEG Effect" was observed with only some of the phenolic compounds. A concrete link between intermediates, enzyme, and substrate for predicting the "PEG effect" was still not determined and thus it is impossible to predict what enzyme-substrate combinations will benefit from additives such as PEG. Despite a PEG effect being observed it is still much smaller than that observed with some of the peroxidases and the findings in this study indicate that it a lower degree of inactivation may be responsible. Since the crude enzyme stock may have low MW PEG that factor may too influence the PEG to substrate ratio. Laccase was capable of reducing the phenol concentration in authentic refinery wastewater although it was not as efficient as the synthetic samples. Further investigation is warranted for fungal laccases for the enzymatic treatment of industrial wastewater effluents.

Chapter 7: Recommendations

Before enzymatic treatment using laccase SP 504 can be implemented into a practical or competitive alternative to existing wastewater treatment methods a number of parameters are left to be assessed.

1. Toxicity of both soluble and insoluble products will have to be further assessed. While previous studies with similar compounds suggest these products are less toxic a more in-depth investigation is warranted. In addition, environmental impact studies should be conducted to determine the fate of these products.
2. More comprehensive product identification studies, including intermediates generated, is required, as well as the effect of multiple substrate mixtures, since effluents rarely consist of one compound. Multiple substrates will greatly expand and diversify the type of products generated. Also, the feasibility of recycling these products should be determined, as opposed to disposal.
3. Oxygen kinetic studies. Detailed oxygen kinetics will be essential to reactor design, especially if it has to be aerated due to higher oxygen demands with increasing pollutant concentration. Oxygen consumption in the presence of compounds competing for oxidant (reducing anions) may also have to be characterized.
4. Alternative coagulation or sedimentation techniques should be investigated. Coloured products are generated and while most are removed through micro-filtration this is not a viable alternative on an industrial scale.
5. More wastewater trials should be conducted, some with higher pollutant concentration, ideally effluents with multiple phenols at high concentrations.

6. Adaptation of enzymatic treatment to continuous-flow type reactors should be examined.
7. Cost analysis in comparison to other enzymes tested should be conducted. Could this enzyme be produced cheap enough to meet large industrial scale demands?
8. Could the enzyme be modified to be more reactive and convert an even broader range of substrates?
9. Mixed substrate reactors and the effect that co-polymerization might have on the level of conversion should be considered.
10. Quantify C-O and C-C coupling with and without additives under various conditions should be quantified.

References

Aasa R., Branden R., Deinum J., Malmstrom B.G., Vanngard T. **(1976)** A paramagnetic intermediate in the reduction of oxygen by reduced laccase. ***FEBS Letters*** 32:187-189.

Akhatar S., Husain Q. **(2006)** Potential applications of immobilized bitter gourd (*Momordica charantia*) peroxidase in the removal of phenols from polluted water. ***Chemosphere*** 65:1228-1235.

Al-Kassim L., Taylor K.E., Nicell J.A. Bewtra J.K., Biswas N., **(1994)** Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxidase from *Coprinus macrorhizus* in batch reactors. ***Journal of Chemical Technology and Biotechnology*** 61:172-181.

Altschuh J., Bruggemann R., Santl H., Eichinger G., Piringer O. **(1999)** Henry's Law constants for diverse set of organic chemicals: Experimental determination and comparison methods. ***Chemospher***e 39: 1871-1997

Aray Y., Marquez M., Rodriguez J., Vega D., Simon-Manso Y., Coll S., Gonzalez C., Weitz D.A. **(2004)** Electrostatics for exploring the nature of the hydrogen bonding in polyethylene oxide hydration. ***Journal of Physical Chemistry B*** 108:2418-2424.

Atlow S.T., Bonadonna-Aparo L., Klibanov A.M. **(1984)** Dephenolization of industrial wastewaters catalyzed by polyphenol oxidase. ***Biotechnology and Bioengineering*** 26:599-603.

Bai J., Wen J-P., Li H-M., Jiang Y. **(2007)** Kinetic modeling of growth and bidegredation of phenol and m-cresol using *Alcaligenes faecalis*. ***Process Biochemistry*** 42:510-517.

Barton S.C., Kim H.-H.. Binyamin G., Zhang Y., Heller A. **(2001)** The "wired" laccase cathode: high current density electroreduction of O_2 + Water at + 0.7V (NHE) at pH 5. ***Journal of American Chemical Society*** 123: 5802-5803.

Barton S.C., Gallaway J., Atanassov P. **(2004)** Enzymatic biofuel cells for implantable and microscale devices. ***Chemical Reiews*** 104:4867-4886.

Bassi A., Geng Z., Gijzen M. **(2004)** Enzymatic removal of phenol and chlorophenols using soybean seed hulls. ***Engineering and Life Science*** 4:125-130.

Beltran F.J., Encinar J.M., Garcia Araya J.F. **(1990)** Ozonation of o-cresol in aqueous solutions. ***Water Research*** 24:1309-1316.

Bento I., Matins L.D., Lopes G.G., Carrondo M.M., Lindley P.F. **(2005)** Dioxygen reduction by multi-copper oxidases structural perspective. ***Dalton Transactions*** 21: 3507-3513.

Bertrand T., Jolivalt C., Briozzo P., Caminade E., Joly N., Madzak C., Mougin C. **(2002)** Crystal structure of a four-copper laccase complexed with an arylamine: insights into substrate recognition and correlation with kinetics. ***Biochemistry*** 41:7325-7333.

Beszedits S., Silbert M.D. **(1990)** Treatment of phenolic wastewater. B & L information services Toronto Canada.

Bodalo A., Gomez J.L., Gomez E., Bastida J., Maximo M.F. **(2006)** Comparison of commercial peroxidases for removing phenol from wastewater solutions. ***Chemosphere*** 63:626-632.

Bollag J.M., Sjoblad R.D., Liu S.Y. **(1979)** Characterization of an enzyme from *Rhizoctionia practicola* which polymerizes phenolic compounds. ***Canadian Journal of Microbiology*** 24:229-233.

Boyd S.A., Shelton D.R., Berry D., Tiedje J.M. **(1983)** Anaerobic biodegradation of phenolic compounds in digested sludge. ***Applied and Environmental Microbiology*** 46:50-54

Branden R. Deinum J. **(1978)** The effect of pH on the oxygen intermediate and the dioxygen reducing site in blue oxidases. ***Biochimica et Biophysica Acta*** 524:297-304.

Branden R. Deinum J., Cleman M. **(1978)** A mass spectrometric investigation of reaction between $^{18}O_2$ and reduced tree laccase a differentiation between the two water molecules formed. ***FEBS Letters*** 89:180-182

Briggs G.G. **(1981)** Theoretical and experimental relationships between soil adsorption, octanol-water partition coefficients, water solubilities, bioconcentration factors and the parachlor. ***Journal of Agriculture and Food Chemistry*** 29:1050-1059

Bukh C., Lund M., Bjerumm M.J. **(2006)** Kinetic studies on the reaction between *Trametes villosa* laccase and dioxygen. ***Journal of Inorganic Biochemistry*** 100:1547-1557.

Busca G., Berardinelli S., Resini C. Arrighi L. **(2008)** Technologies for the removal of phenol from fluid streams: A short review of recent developments. ***Journal of Hazardous Materials*** DOI:10.1016/j.jhazmat.2008.03.045.

Carvalho M.C., Pereira C., Goncalves I.C., Pinheiro H.M., Santos A.R., Lopes A., Ferra M.I., **(2008)** Assesment of the biodegradability of monosulfonated azo dye and aromatic amines. ***International Biodeterioration & Biodegradation*** DOI:10.1016/j.j.ibiod.2007.12.008.

Caza, N. Bewtra J.K., Biswas N., Taylor K. E. **(1999)** Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. ***Water Research*** 33:3012-3018.

Chen G. **(2004)** Electrochemical technologies in wastewater treatment. ***Separation and Purification Technology*** 38:11-41.

Clark P.A., Solomon E.I. **(1992)** Magnetic circular dichroism spectroscopic definition of the intermediate produced in the reduction of dioxygen to water by native laccase. ***Journal of the American Chemical Society*** 114:1108-1110.

Cole J. L., Ballou D.P., Solomon E.I. **(1991)** Spectroscopic characterization of the peroxide intermediate in the reduction of dioxygen catalyzed by the multicopper oxidases. ***Journal of the American Chemical Society*** 113:8544-8546.

Cooper R.L., Wheatstone K.C. **(1973)** The determination of phenols in aqueous effluents ***Water Research*** 7:1375-1384.

Cruz-Silva R., Romarero-Garcia J., Angulo-Sanchez J.L., Ledezma-Perez A., Arias-Marin E., Moggio I., Flores-Loyola E. **(2005)** Template-free enzymatic synthesis of electrically conducting polyaniline using soybean peroxidase. ***European Polymer Journal*** 41:1129-1135.

Decker Heinz, Schweikardt T., Nillus D., Salzbrunn U., Jaenicke, Tuczek F. **(2007)** Similar enzyme activation and catalysis in hemocyanins and tyrosinases. ***Gene*** 398:183-191.

De Souza C.G.M., Tychanowicz G.K., De Souza D.F., Pertalta R.M. **(2004)** Production of laccase isoforms by *Pleurotus pulmonarius* in response to presence of phenolic and aromatic compounds. ***The of Journal Basic Microbiology*** 44:129-136.

Dong J.L., Zhang Y.W., Zhang R.H, Haung W.Z., Zhang Y.Z. **(2005)** Influence of culture conditions on laccase production and isozyme patterns in the white-rot fungus *Trametes gallica.* ***The Journal of Basic Microbiology*** 45:190-198.

Duarte-Vazquez M.A., Ortega-Tovar M.A., Garcia-Almendarez B.E. **(2002)** Removal of aqueous phenolic compounds from a model system by oxidative polymerization with turnip (*Brassica napus* L var purple top white globe) peroxidase**. Journal of Chemical Technology and Biotechnology** 78:42-47.

Ducros V., Brzozowski A.M., Wilson K.S., Brown S.H., Davies G.J. **(1998)** Crystal structure of the type-2 depleted laccase from *Coprinus cinereus* at 2.2Å resolution. ***Nature Structural and Molecular Biology.*** 5:310-316.

Dunford H.B. **(1999)** Heme Peroxidases. John Wiley & Sons Inc., New York, pp 1-57, 299-304.

Ellis D.D. Jone C.M., Larson R.A., Schaeffer D.J. **(1982)** Organic constituents of mutagenic secondary effluents from wastewater treatment plants. ***Archives of Environmental Contamination and Toxicology*** 11:373-382.

EPA-TRI Environmental Protection Agency – Toxic Release Inventory (**2008**), website: http://www.epa.gov/tri Accessed June, 2008.

Farmer J.C., Wang F.T., Hawley-Fedder R.A., Lewis P.R., Summers R.J., Foiles L. (**1992**) Electrochemical treatment of mixed and hazardous wastes:oxidation of ethylene glycol and benzene by silver(II). ***Journal of Electrochemical Society*** 139:654-662.

Faust S.D., Mikulewicz E.W. (**1967**) Factors influencing the condensation of 4-aminoantipyrine with derivatives of hydroxybenzene-II. Influence of hydronium ion concentration on absorptivity. ***Water Research*** 1:509-522.

Fedorak P.M., Hrudey S.E. (**1986**) Anaerobic treatment of phenolic coal conversion wastewater in semicontinious cultures. ***Water Research*** 20:113-122.

Felby C. (**1998**) Measurment of laccase activity by a syringaldazine based assay. Application Development Novo Nordisk.

Gaber K., Harreus U.A., Matthias C., Kleinsasser N.H., Richter E. (**2007**) Hemoglobin adducts of the human bladder carcinogen o-toludine after treatment with the local anethetic priocaine. ***Toxicology*** 229:157-164.

Gaffney J.S., Streit G.E., Spall W.D., Hall J.H. (**1987**) Beyond Acid Rain: Do soluble oxidants and organic toxins interact with SO_2 and NO_x to increase ecosystem effluents? ***Environmental Science and Technology*** 21:519-525

Galhaup C., Haltrich D. (**2001**) Enhanced formation of laccase activity by the white-rot fungud *Trametes pubescens* in the presence of copper. ***Applied Microbiology and Biotechnology*** 56:225-232.

Galli C., Gentili P., Nunes Pontes A.S., Gamelas J.A.F., Evtuguin D.V. (**2007**) Oxidation of phenols employing polyoxometaletes as biomimetic models of the activity of phenoloxidase enzymes ***New Journal of Chemistry*** 31:1461-1467.

Gawlik B.M., Feicht E.A., Karcher W., Kettrup A., Muntau H. (**1998**) Application of the European Reference Soil Set (EUROSOILS) To a HPLC-screening method for the estimation of soil adsorption coefficients of organic compounds. ***Chemosphere*** 36: 2903-2919.

Geng X., Li K., Xu F. (**2004**) Investigation of hydroxamic acids as laccase-mediators for pulp bleaching. ***Applied Microbiology and Biotechnology*** 64:493-496.

Ghosh J. P., Taylor K. E. Bewtra J. K., Biswas N. (**2008**) Laccase-catalyzed removal of 2,4-dimethylphenol from synthetic wastewater: Effect of polyethylene glycol and dissolved oxygen. ***Chemosphere*** 71:1709-1717.

Ghindilis A.L., Garilova V.P., Yaropolov A.I. **(1992)** Laccase-based biosensor for determination of polyphenols: determination of catechols in tea. ***Biosensors and Bioelectronics*** 7:127-131.

Ghioureliotis M., Nicell J. A. **(1999)** Assesment of soluble products of peroxidase-catalyzed polymerization of aqueous phenol. ***Enzyme and Microbial Technology*** 25:185-193.

Gomes S.A.S.S., Nogueira J.M.F., Rebelo M.J.F. **(2004)** An amperometric biosensor for polyphenolic compounds in red wine. **Biosensors and Bioelectronics** 20:1211-1216.

Gonzalez P.S., Agostini E., Milrad S.R. **(2008)** Comparison of the removal of 2,4-dichlorophenol and phenol from polluted water, by peroxidases from tomato hairy roots, and protective effect of polyethylene glycol. ***Chemosphere*** 70:982-989.

Gomori G., Preparation of buffers for use in enzyme studies. **(1955)** In Colowick S.P., Kaplan N.O. (Eds.), Methods in Enzymology. New York: Academic Press Inc., 138-146.

Gottlieb H.E., Kotlyar V., Nudelman A. **(1997)** NMR chemical shifts of common laboratory solvents as trace impurities. ***Journal of Organic Chemistry*** 62:7512-7515.

Graham N., Jiang C.C., Li XZ., Jiang J.Q., Ma J. **(2004)** The influence of pH on the degradation of phenol and chlorophenols by potassium ferrate. ***Chemosphere*** 56:949-956.

Gu X., Zhou J., Zhang A., Wang P., Xiao M., Guangfei L. (2008) Feasibility study of the treatment of aniline hypersaline wastewater with a combined adsorption/bio-regeneration system. ***Desalination*** 227: 139-149.

Hamoudi S., Sayari A., Belkacemi K., Bonneviot I., Larachi F. **(2000)** Catalytic wet oxidation of phenol over Pt_xAg_{1-x}-MnO_2/CeO_2 catalyst. ***Catalyst Today*** 62:379-388.

Hansch C., Leo A.D., Hoekman **(1995)** Exploring QSAR hydrophobic, electronic and steric constants. Washington, DC, American Chemical Society pg 32.

Hatvani N., Mecs I. **(2003)**. Effects of certain heavy metals on the growth, dye decolorization, and enzyme activity of *Lentinula edodes*. ***Ecotoxicology and Environmental Safety*** 55:199-203.

Huang H.-W., Zoppellaro G., Sakurai T. **(1999)** Spectroscopic and kinetic studies in the oxygen-centered radical formed during the four-electron reduction process of dioxygen by *Rhus vernicifera* laccase. ***The Journal of Biological Chemistry*** 274:32718-32724.

Huang L., Nishinari K. **(2001)** Interaction between poly(ethylene glycol) and water as studied by differential scanning calorimetry. ***Journal of Polymer Science B: Polymer Physics*** 39:496-506

Huang Q. G., Weber W.J. Jr. **(2005)** Transformation and removal of bisphenol A from aqueous phase via peroxidase-mediated oxidative coupling reactions: efficacy, products, and pathways. **Environmental Science and Technology** 39:6029-6036.

Ibrahim M.S., Ali H. I., Taylor K.E., Biswas N., Bewtra J.K., **(2001)** Enzyme catalyzed removal of phenol from refinery wastewater; feasibility studies. ***Water Environment Research*** 73:165-172.

Ikehata K. Nicell J.A. **(2000)** Characterization of tyrosinase for the treatment of aqueous phenols. ***Bioresource Technology*** 74:191-199.

Ikehata K., Buchanan I.D., Pickard M.A., Smith D.W. **(2005)** Purification, characterization and evaluation of extracellular peroxidase from two *Coprinus* species for aqueous phenol treatment. ***Bioresource Technology*** 96:1758-1770.

Ikehata K., Buchanan I.D. **(2002)** Screening of *Coprinus* species for the production of extracellular peroxidase and evaluation of the enzyme for the treatment of aqueous phenol. ***Environmental Technology*** 23:1355-1368.

Jayasinghe D.S., Brownawell B.J. Chen H. Westall J.C. **(1992)** Determination of Henry's constant of organic compounds of low volatility: methylanilines in methanol and water. ***Environmental Science and Technology*** 26:2275-2781

Juang R.S., Tsai S.Y. **(2006)** Growth kinetics of *Pseudomonas putida* in the biodegradation of single and mixed phenol and sodium salicylate. ***Biochemical Engineering Journal*** 31:133-140.

Karamyshev A.V., Shleev S.V., Koroleva O.V., Yaropolov A.I., Sakharov I.Y. **(2003)** Laccase-catalyzed synthesis of conducting polyaniline. ***Enzyme and Microbial Technology*** 33:556-564.

Karlsson B.G., Aasa R., Malmstrom B.G., Lundburg L.G. **(1989)** Rack-induced bonding in blue copper proteins: spectroscopic properties and reduction potential of the azurin mutant. ***FEBS Letters*** 253:99-102.

Kavitha V., Palanivelu K., **(2005)** Destruction of cresols by Fenton oxidation process. ***Water Research*** 39:3062-3072.

Kim Y-J,, Uyama H., Kobayashi S. **(2003)** Regioselective synthesis of Poly(phenylene) as a complex with Poly(ethylene glycol) by template polymerization of phenol in water. ***Macromolecules*** 36:5058-5060.

Kim Y.-J., Uyama H., Kobayashi S. **(2004)** Peroxidase-catalyzed oxidative polymerization of phenol with a non-ionic polymer surfactant template in water. ***Macromolecular Bioscience*** 4:497-502.

Kim Y.-J., Nicell J. A. **(2006)** Impact of reaction conditions on the laccase-catalyzed conversion of bisphenol A. ***Bioresource Technology*** 97:1431-1442.

Kim Y.-J., Nicell J.A. **(2006a)** Laccase-catalyzed oxidation of bisphenol A with the aid of additives. ***Process Biochemistry*** 41:1029-1037.

Kinsley C., Nicell J.A., **(2000)** Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol. ***Bioresource Technology*** 73:139-146.

Kjellander R., Florin E. **(1981)** Water structure and changes in thermal stability of the system poly(ethylene oxide) water. ***Journal of the Chemical Society, Faraday Transaction I.*** 77:2053-2077

Klibanov A. M., Morris E.D. **(1980)** Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. ***Enzyme and Microbial Technology*** 3:119-122.

Klibanov A. M., Alberti B. N., Morris E.D., Felshin L.M. **(1980)** Enzymatic removal of toxic phenols and anilines from waste waters. ***Journal of Applied Biochemistry*** 2:414-421.

Klibanov A.M., Tu T. Scott K.P. **(1983)** Peroxidase-catalyzed removal of phenols from coal-conversion wastewaters. ***Science*** 221:259-261.

Ko E.-M., Leem Y.-E., Choi H.T. **(2001)** Purification and characterization of laccase isozymes from the white-rot basidomycete *Ganadenna lucidum*. ***Applied Microbiology and Biotechnology*** 57:98-102.

Kopinke F.D. Poerschmann J., Stottmeister U. **(1995)** Sorption of organic pollutants on anthropogenic humic matter. ***Environmental Science and Technology*** 29:941-950

Kurniawati S. Nicell J.A. **(2008)** Characterization of *Trametes versicolor* laccase for the transformation of aqueous phenol. ***Bioresource Technology*** doi:10.1016/j.biotech.2008.01.084

Kuyls J., Viziunaite R. **(2002)** Modulation of laccases activity by soluble polymers. ***Biologija*** 2: 3-6 (ISSN 1392-0146).

Kulys J. Vidziunaite R., Schneider P., **(2003)** Laccase-catalyzed oxidation of napthol in the presence of soluble polymers. ***Enzyme and Microbial Technology*** 32:455-463.

Kumar S.V.S. Phale P.S., Durani S., Wangikar P.P. **(2003)** Combined sequence and structure analysis of the fungal laccase family. ***Biotechnology and Bioengineering*** 83:386-394.

Laszlo K. **(2005)** Adsorption from aqueous phenol and aniline solutions on activated carbons with different surface chemistry. ***Colloids and Surfaces A: Physical Chemistry and Engineering Aspects*** 265:32-39.

Lee S.-K., George S.D., Antholine W.E., Hedman B., Hodgson K.O., Solomon E.I. **(2002)** Nature of the intermediate formed in the reduction of O_2 to H_2O at the trinuclear copper cluster active site in native laccase. ***Journal of American Chemical Society*** 124:6180-6193.

Lee Y., Yoon J., von Gunten U. **(2005)** Kinetics of the oxidation of phenols and phenolic endocrine disruptors during water treatment with ferrate (Fe(IV)). ***Environmental Scinece and Technology*** 39:8978-8984.

Li X.Y., Chi Y.H., Feng Y.J., Xie Z.M., Gu J.D. **(2005)** Reaction pathways and mechanisms of the electrochemical degradation of phenol on different electrodes. ***Water Research*** 39:1972-1981.

Lide D.R., (ed.) **(2002-2003)** CRC Handbook of Chemistry and Physics. 83rd edition, Boca Raton, Fl, CRC Press Inc.

Lin-Gibson S., Baranauskas V., Riffle J.S., Sorathia U. **(2002)** Cresol novolac – epoxy networks: properties and processability. ***Polymer*** 43:7389-7398.

Litthauer D., van Vuuren M.J., van Tonder A., Wolfaardt F.W. **(2007)** Purification and kinetics of a thermostable laccase from *Pycnoporus sanguineus* (SCC 108). **Enzyme and Microbial Technology** 40:563-568.

Lorenzo M., Moldes D., Couto R. S., Sanroman M[a] A. **(2005)** Inhibition of laccase activity from *Trametes versicolor* by heavy metals and organic compounds. ***Chemosphere*** 60:1124-1128.

Machonkin T.E., Solomon E.I. **(2000)** The Thermodynamics, kinetics, and molecular mechanism of intermolecular electron transfer in human ceruloplasmin. ***Journal of American Chemical Society*** 122:12547-12560.

Madzak C., Mimmi M. C., Caminade E., Brault A., Baumberger S., Briozzo P., Mougin C., Jolivalt C. **(2006)** Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. ***Protein Engineering Design & Selection*** 19:77-84.

Malmstrom Bo. G. **(1997)** Chapter 6: A life with the metals of life. ***Comprehensive Biochemistry*** 40: 277-331.

Matera I. Gullotto A. Tilli S., Ferraroni M., Scozzafava A., Briganti F. **(2008)** Crystal structure of the blue multicopper oxidase from the white-rot fungus *Trametes trogii* complexed with p-toluate. ***Inorganica Chimica Acta*** DOI:10.1016/j.ca.2008.03.091.

Means G,E., Congdon W.I., Bender M.L. **(1972)** Reactions of 2,4,6-trinitrobenzenesulfonate ion with amines and hydroxide ions. ***Biochemistry*** 11:3564-3571.

Mita N., Tawaki S.I., Uyama H., Kobayashi S. **(2003)** Laccase-catalyzed oxidative polymerizationof phenols. ***Macromolecular Bioscience*** 3:253-257.

Modaressi K., Taylor K. E., Bewtra J. K., Biswas N. **(2005)** Laccase-catalyzed removal of bisphenol A from water: Protective effect of PEG on enzyme activity. ***Water Research*** 39: 4309-4316.

Modaressi K., **(2007)** Enzyme catalyzed polymerization and removal of bisphenol A from wasterwater with laccase. (PhD. Dissertation, University of Windsor, Windsor, ON.)

Morozova O.V., Shumakovich G.P., Gorbacheva M. A., Shleev S.V., Yaropolov A.I. **(2007)** "Blue" Laccases. ***Biochemistry (Moscow)*** 72:1136-1150.

Mousa M. **(2008)** Removal of various aromatic compounds from synthetic and refinery wastewater using soybean peroxidase. (M.Sc. Thesis, University of Windsor, Windsor ON.).

Munoz C., Guillen G., Martinez A.T., Martinez M.J. **(1997)** Laccase isozymes of *Pleurotus eryngii* characterization, catalytic properties and participation in activation of molecular oxygen and Mn^{2+} oxidation. ***Applied and Environmental Microbiology*** 63:2166-2174

Nakamoto, S., Machida N. **(1992)** Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. ***Water Research*** 26:49-54.

Nakhla G.F., Suidan M.T. **(1995)** Anaerobic toxic wastes treatment dilution effects. ***Journal of Hazardous Materials*** 42:71-86.

Nicell J.A., Al-Kassim L., Bewtra J.K., Taylor K.E. **(1993)** Wastewater treatment by enzyme catalyzed polymerization and precipitation. ***Biodeterioration Abstracts*** 7:1-8.

Niedermeyer T.H.J., Lalk M. **(2007)** Nuclear amination catalyzed by fungal laccases: Comparison of laccase catalyzed amination with known chemical routes to aminoquinones. ***Journal of Molecular Catalysis B: Enzymatic*** 45:113-117.

Niladevi K.N., Jacob N., Prema P. **(2008)** Evidence for a halotolerant-alkaline laccase in *Streptomyces psammoticus*: Purification and characterization. ***Process Biochemistry*** 43:654-660.

NPRI – National pollutant release inventory (**2008**), website: http://www.ec.gc.ca/pdb/npri_home_e.cfm Accessed June, 2008.

Oguchi T., Wakisaka A., Tawaki S.-I., Tonami H., Uyama H., Kobayashi S. (**2002**) Self-association of m-cresol in aqueous organic solvents: Relation to enzymatic polymerization reaction. ***Jouranl of Physical Chemistry B*** 106:1421-1429.

O'Niel F.J., Bromley Challenor K.C.A., Greenwood R.J., Knapp J.S. (**2000**) Bacterial growth on aniline: implications for the biotreatment of industrial wastewater. ***Water Research*** 34:4397-4409.

O'Neil M.J. (Ed) (**2001**) The Merck Index – An encyclopedia of chemical and drugs and biologicals 13th Edition, Whitehouse Station NJ, Merk & Co. Inc.

Palmer A.E., Lee S.K., Solomon E.I. (**2001**) Decay of peroxide intermediate in laccase: reductive cleavage of the O-O bond. ***Journal of American Chemical Society*** 123:6591-6599.

Palmer A.E., Randall D.W., Xu F., Solomon E.I. (**1999**) Spectoscopic studies and electronic structure description of the high potential type 1 Cu site in fungal laccase: Insight into the effect of the axial ligand ***Journal of American Chemical Society*** 121:7138-7149.

Palmieri G., Cennamo G., Faraco V., Amoresano A., Sannia G., Giardina P. (**2003**) Atypical laccase isoenzymes from copper supplemented *Pleurotus ostreatus* cultures ***Enzyme and Microbial Technology*** 33:220-230

Panizza M., Cerisola G. (**2001**) Removal of organic pollutants from industrial wastewater by electrogenerated Fenton's reagent. ***Water Research*** 35:3987-3992.

Panizza M., Cerisola G., (**2005**) Application of diamone electrodes to electrochemical processes. ***Electrochim Acta*** 51:191-1999.

Pascher T., Karlsson B.G., Nordling M., Malmstrom B.G., Vanngard T. (**1993**) Reduction potential and their pH dependence in site-directed-mutant forms of azurin from *Pseudomonus aeruginosa*. ***European Journal of Biochemistry*** 212:289-296.

Patapas J., Al-Ansari M.M., Taylor K.E., Bewtra J.K, Biswas N. (**2007**) Removal of dinitrotoluenes from water via reduction with iron and peroxidase-catalyzed oxidative polymerization: A comparison between Arthromyces ramous peroxidase and soybean peroxidase. ***Chemosphere*** 67:1485-1491.

Pearce P.S., Simkins R.J.J. (**1968**) Acid strengths of some substituted picric acids ***Canadian Journal of Chemistry*** 46:241-248.

Piontek K. Antorini M., Choinowski T. **(2002)** Crystal Structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. ***Journal of Biological Chemistry*** 277:37663-37669.

Polowinski S. **(2002)** Template polymerization and co-polymerization. ***Progress in Polymer Science*** 27:537-577.

Qi X.-H., Zhuang Y.-Y., Yuan Y.-C., Gu W.-X. **(2002)** Decomposition of aniline in supercritical water. ***Journal of Hazardous Materials*** 90:51-62.

Quintanat L., Yoon J., Aznar C.P., Palmer A.E., Anderson K.K., Britt R.D., Solomon E.I. **(2005)** Spectroscopic and electronic structure studies of the trinuclear Cu cluster active site of the multicopper oxidase laccase: Nature of its coordination unsaturation ***The Journal of American Chemical Society*** 127:13832-13845.

Quintanilla-Guerrero F., Duarte-Vazquez M.A., Garcia-Almendarez B.E., Tinoco R., Vazquez-Duhalt R., Regalado C. **(2008)** Polyethylene glycol improves phenol removal by immobilized turnip peroxidase. ***Bioresource Technology*** DOI:10.1016/j.biortech.2008.04.031.

Reinhammer B.R., Vanngard T.I. **(1971)** The electron-accepting sites in *Rhus vernicifera* laccase as studied by anaerobic oxidation-reduction titrations. ***European Journal of Biochemistry*** 18:463-468.

Reinhammer B.R., **(1972)** Oxidation-reduction potentials of the electron acceptors in laccase and stellacyanin. ***Biochimica et Biophysica Acta*** 275:245-259.

Reinhammer B. Malkin R. Jensen P., Karlsson B., Andreasson L.E., Aasa R., Vanngard T., Malmstrom B.G **(1980)** A new copper (II) electron paramagnetic resonance signal in two lacasses and in cytochrome c oxidase. ***Journal of Biological Chemistry*** 255:5000-5003.

Reinhammer B. **(1981)** An epr signal from the half-reduced type 3 copper pair in *Rhus vernicifera* laccase. ***Journal of Inorganic Biochemistry*** 15:27-39.

Riva S. **(2006)** Laccases: Blue enzymes for green chemistry. **Trends in Biotechnology** 24:219-226.

Rodakiewicz-Norwak J., Kasture S.M., Dudek B., Haber J. **(2000)** Effect of various water-miscible solvents on enzymatic activity of fungal laccases. **Journal of Molecular Catalysis B: Enzymatic** 11:1-11.

Ryan D.R., Leukes W.D., Burton S.G. **(2005)** Fungal bioremediation of phenolics wastewaters in an airlift reactor. ***Biotechnology Progress*** 21:1066-1074.

Sato T., Niwa H., Chiba A., Nozaki R., **(1998)** Dynamic structure of oligo(ethylene glycol)s – water solutions studied by time domain reflectrometry. ***Journal of Chemical Physics*** 108:4138-4147

Santos A., Yustos P., Rodriguez S., Garcia-Ochoa F. **(2006)** Wet oxidation of phenol, cresols and nitrophenols catalyzed by activated carbon in acidand basic media. ***Applied Catalysis B: Environmental*** 65:269-281.

Saravanan P., Pakshirajan K., Saha P., **(2008)** Biodegredation of phenol and m-cresol in a batch and fed batch operated internal loop airlift bioreactor by indigenous mixed microbial culture predominatly *Pseudomonas sp.* ***Bioresource Technology*** DOI:10.1016/j.biotch.2008.04.003.

Saha B. **(2006)** Laccase catalyzed removal of diphenylamine from synthetic wastewater. (M.A.Sc. Thesis, University of Windsor, Windsor ON.).

Scott H.D., Wolf D.C., Lavy T.L. **(1983)** Adsorption and degradation of phenol at low concentrations in soil. ***Journal of Environmental Quality*** 12:91-95.

Shackelford W.M. Cline D.M., Faas L., Kurth G. **(1983)** An evaluation of automated spectrum matching for survey identification of wastewater components by gas chromatography-mass spectrometry. ***Analytica Chimica Acta*** 146:15-27.

Shleev S., Christenson A., Serezhenkov V., Burbaev D., Yaropopov A., Gorton L., Ruzgas T. **(2005)** Electrochemical redox transformations of T1 and T2 copper sites in native *Trametes hirsute* laccase at gold electrode. ***Biochemistry Journal*** 385:745-754.

Shleev S., Reimann C.T., Serezhenkov V., Burbaev D., Yaropolov A.I., Gorton L., Ruzgas T. **(2006)** Autoreduction and aggregation of fungal laccase in solution phase: possible correlation with resting form of laccase. ***Biochimie*** 88:1275-1285.

Shleev S., Persson P., Shumakovich G., Mazhugo Y., Yaropolov A., Ruzgas T., Gorton L. **(2006)** Laccase-based biosensors for monitoring lignin. ***Enzyme and Microbial Technology*** 39:835-840.

Shikata T., Takahashi R., Sakamoto A. **(2006)** Hydration of poly(ethylene oxide)s in aqueous solution as studied by dielectric relaxation measurments. ***Journal of Physical Chemistry B*** 110:8941-8945.

Shin W., Sundaram U.M., Cole J.L., Zhang H.H. Hedman B., Hodgson K.O., Solomon E.I. **(1996)** Chemical and spectroscopic definition of the peroxide-level intermediate in the multicopper oxidases: Relevance to the catalytic mechanism of dioxygen reduction to water. ***Journal of American Chemical Society*** 118:3202-3215.

Shiu W.Y., Ma K.-C., Varhanickova D., Mackay D. **(1994)** Chlorophenols and alkylphenols: A review and correlation of environmentally relevant properties and fate in evaluative environment. ***Chemosphere: Global Change Science*** 29:1155-1224.

Singh R.K., Kumar S., Kumar S., Kumar A. (2008) Development of parthenium based activated carbon and its utilization for adsorptive removal of p-cresol from aqueous solution. **Journal of Hazardous Materials** 155:523-535.

Smirnov S.A., Koroleva O.V., Gavriloa V.P., Belova A.B., Klyachko N.L. **(2001)** Laccase from bacidiomycetes: physical characteristics and substrate specifity towards methoxyphenolic compounds. ***Biochemistry (Moscow)*** 66:774-779.

Solomon E.I., Sundaram U.M., Machonkin T.E. **(1996)** Multicopper oxidases and oxygenases. ***Chemical Reviews*** 96:2563-2605.

Steevensz A. Al Ansari M. M., Taylor K.E., Bewtra J.K., Biswas N. **(2008)** Comparison of soybean peroxidase with laccase in the removal of phenol from synthetic and refinery wastewaters. ***Submitted.***

Suarez-Ojeda E.M., Carrera J., Metcalfe I.S., Font J. **(2008)** Wet air oxidation (WAO) as a precursor to biological treatment of substituted phenols : Refactory nature of the WAO intermediates. ***Chemical Engineering Journal*** DOI:10.1016/j.cej.2008.01.022.

Swann R.L., Laskowski D.A., McCall P.J., Van der Kuy K., Dishburger H.S. **(1983)** A rapid method for the estimation of the environmental parameters octanol/water partition coefficient, soil sorption constant, water to air ratio and water solubility. Research Reviews 85:17-28

Tadesse M. A., D'Annibale A. Galli C., Gentilli P., Sergi F. **(2008)** An assessment of the relative contributions of redox and steric issues to laccase specifity towards putative substrates. ***Organic & Biomolecular Chemistry*** 6:868-878.

Tay J.H., He Y.X., Yan. Y.G. **(2001)** Improved anaerobic degredation of phenol with supplemental glucose. ***Journal of Environmental Engineering*** ASCE, 127:38-45.

Taylor K.E., Al-Kassim L., Bewtra J.K., Biswas N., Taylor J. **(1996)** Enzyme based wastewater treatment: removal of phenols by oxidative enzymes. In ***Environmental Biotechnolog: Principles and Applications,*** M. Moo-Young, W.A. Anderson, A.M. Chakrabarty (editors), Kluwer Academic Publishers, Dordrecht (ISBN 0-7923-3877-4 :524-532.

Taylor K.E., Bewtra J.K., Biswas N. **(1998)** Enzymatic treatment of phenolic and other aromatic compounds in wastewaters. **Water Environment Federation**, 71st Annual Conference & Exposition Proceedings (ISBN 1-57278-138-6) 3:349-360.

Thakker G., Evans C.S., Rao K.K. **(1992)** Purification and characterization of laccase from Monocillium indicum Saxena. ***Applied Microbiology and Biotechnology*** 37:321-323.

Thompson D.C., Perera K., Fisher R., Brendel K., **(1994)** Cresol isomers: Comparison of toxic potency in rat liver slices. ***Toxicology and Applied Pharmacology*** 125:51-54.

Thompson T.L., Yates Jr. J.T. **(2006)** Surface science studies of the photoactivation of TiO_2-new photochemical processes. ***Chemical Reviews*** 106-4428-4453.

TOXNET: Toxicology Data Network, Hazardous substance data bank (HSDB) **(2008)**, website: http://toxnet.nlm.nih.gov. Accessed June, 2008.

Treetharnmathurot B., Overtlarnporn C., Wungsintaweekul J., Duncan R., Wiwattanapatapee R. **(2008)** Effect of PEG molecular weight and linking chemistry on the biological activity and thermal stability of PEGylated trypsin. ***International Journal of Pharmaceutics*** 357:252-259.

Veeresh G.S., Kumar P., Mehrotra I. **(2005)** Treatment of phenol and cresols in upflow anaerobic sludge blanket (UASB) process: a review. ***Water Research*** 39:154-170.

Vermette A. **(2000)** Removal of cresols from synthetic wastewater using laccase. (M.A.Sc. Thesis, University of Windsor, Windsor ON.).

Villalobos D.A., Buchanan I.D. **(2002)** Removal of aqueous phenol by *Arthromyces ramosus* peroxidase. ***Journal of Environmental Engineering Science***. 1:65-73.

Wada S., Ichikawa H., Tatsumi K., **(1993)** Removal of phenols from wastewater by soluble and immobilized tyrosinase. ***Biotechnology and Bioengineering*** 42:854-858.

Wada S., Ichikawa H., Tatsumi K. **(1995)** Removal of phenols and aromatic amines from wastewaterby a combination treatment with tyrosinase and a coagulant. ***Biotechnology and Bioengineering*** 45:304-309.

Wagner M., Nicell J.A. **(2002)** Impact of dissolved wastewater constituents on peroxidase-catalyzed treatment of phenol. ***Journal of Chemical Technology and Biotechnology*** 77:419-428.

Wang L., Barrington S., Kim J.-W. **(2007)** Biodegradation of pentyl amine and aniline from petrochemical wastewater. ***Journal of Environmental Management*** 83:191-197.

Wang L., Zheng L.X., **(1999)** The research of CBDS system simulation on petrochemical wastewater treatment process. ***Environmental Protection*** 260:32-39.

Wang L., Biswas N., Bewtra J.K., Taylor K.E. **(2005)** A simple colormetric method for analysis of aqueous phenylenediamines and aniline. ***Journal of Environmental Engineering Science*** 4:423-427.

Weinstock I.A. **(1998)** Homogeneous-phase electron-transfer reactions of polyoxometalates. ***Chemical Reviews*** 98:113-170.

Windholz M. (Ed), Budavari S. (Co-Ed), Blumetti R.R. (Associate Ed), Otterbien E.S. (Assistant Ed) **(1983)** The Merck Index – An encyclopedia of chemical and drugs and biologicals 10th Edition, Whitehouse Station NJ, Merk & Co. Inc.

Witayakran S., Gelbaum L., Ragauskas A.J. **(2007)** Cascade synthesis of benzofuran derivatives via laccase oxidation-Michael addition. ***Tetrahedron*** 63:10958-10962.

World Health Organization (WHO) **(1963)** International standards for drinking water, Geneva p. 40.

Wright H., Nicell J.A. **(1999)** Characterization of soybean peroxidase for the treatment of aqueous phenols. ***Bioresource Technology*** 70:69-79.

Wu J., Taylor K.E., Bewtra J.K., Biswas N. **(1993)** Optimization of the reaction conditions for enzymatic removal of phenol from wastewater in the presence of polyethylene glycol. ***Water Research*** 27:1701-1706.

Wu Y., Bewtra J.K., Biswas N., Taylor K.E. **(1993)** Removal of phenol derivatives from aqueous solution by enzymatic reaction with additives. In ***Proceedings of the 48th Purdue Industrial Waste Conference***, Ed. Wukash R.F. pp. 421-431 Lewis Publishers, Chelsea, MI.

Wu Y., Taylor K. E. Biswas N., Bewtra J.K. **(1997)** Comparison of additives in the removal of phenolic compounds by peroxidase-catalyzed polymerization. ***Water Research*** 31:2699-2704

Wu Y., Taylor K. E., Bewtra J. K., Biswas N. **(1998)** A model for the protective effect of additives on the activity of horseradish peroxidase in the removal of phenol. ***Enzyme and Microbial Technology*** 22:315-322.

Wu Y., Taylor K.E., Bewtra J.K., Biswas N., **(1999)** Kinetic model for the removal of phenol by horseradish peroxidase with PEG. ***Journal of Environmental Engineering*** 125:451-458.

Xing-yu L. Bao-jun W., Cheng-ying J., Ke-xin Z., Drake, H.L., Shuang-Jiang L. **(2007)** Simultaneous biodegradation of nitrogen-containing aromatic compounds in a sequencing batch reactor. ***Journal of International Sciences*** 19:530-535.

Xu F. **(1996)** Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition. ***Biochemistry*** 35:7608-7614.

Xu F., Shin W. Brown S.H., Wahleithner J.A., Sundaram U.M., Solomon E.I. **(1996)** A study on a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specifity, ans atability. ***Biochimica et Biophysica Acta*** 1292: 303-311.

Xu F. **(1997)** Effects of redox potential and hydroxide inhibition on the pH activity profile of fungal laccases. ***Journal of Biological Chemistry*** 272: 924-928.

Xu F., Berka R.M., Wahleithner J.A., Nelson B.A., Shuster J.R., Brown S.H., Palmer A E., Solomon E.I. **(1998)** Site-directed mutations in fungal laccase: effect on redox potential, activity and pH profile. ***The Biochemical Journal*** 334:63-70.

Xu F., Palmer A., Yaver D.S., Berka R. M., Gambetta G.A., Brown S.H., Solomon E.I., **(1999)** Targeted mutations in *Trametes villosa* laccase axial perturbations of the T1 Copper. ***The Journal of Biological Chemistry*** 274:12372-12375.

Xu F., Deussen H.-J. W., Lopez B., Lam L., Li K. **(2001)** Enzymatic and electrochemical oxidation of N-hydroxy compounds redox potential, electron transfer kinetics, and radical stability. ***European Journal of Biochemistry*** 268:4169-4176.

Yamada K., Akiba Y., Shibuya T., Kashiwada A., Matsuda K., Hirata M. **(2005)** Water purification through bioconversion of phenol compounds by tyrosinase and chemical adsorbtion by chitosan beads. ***Biotechnology Progress*** 21:823-829.

Yan J., Jiangping W., Bai J., Daoquan W., Zongding H., **(2006)** Phenol biodegradation by the yeast *Candida tropicalis* in the presence of m-cresol. ***Biochemical Engineering Journal*** 29:223-227.

Yaropolov A.I., Skorobogat'ko O.V., Vartanov S.S., Varfolomeyev S.D. **(1994)** Laccase. Properties, catalytic mechanism, and applicability. ***Applied Biochemistry and Biotechnology*** 49:257-280

Yaver D.S., Xu F., Golightly E.J., Brown K.M., Brown S.H., Rey M.W., Schneider P., Halkier T., Mondorf K., Dalboge H. **(1996)** Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomyces *Trametes villosa.* ***Applied Environmental Microbiology*** 62:834-841.

Yoon J., Liboiron B.D., Sarangi R., Hodgson K.O., Hedman B., Solomon E.I. **(2007)** The two oxidized forms of the trinuclear Cu cluster in the multicopper oxidases and mechanism for the decay of the native intermediate. ***Proceedings of the National Academy of Sciences*** (PNAS check) 104:13609-13614.

Yoshida H. **(1883)** Chemistry of lacquer (urushi).***The Journal of Chemistry Society*** 43:472-486.

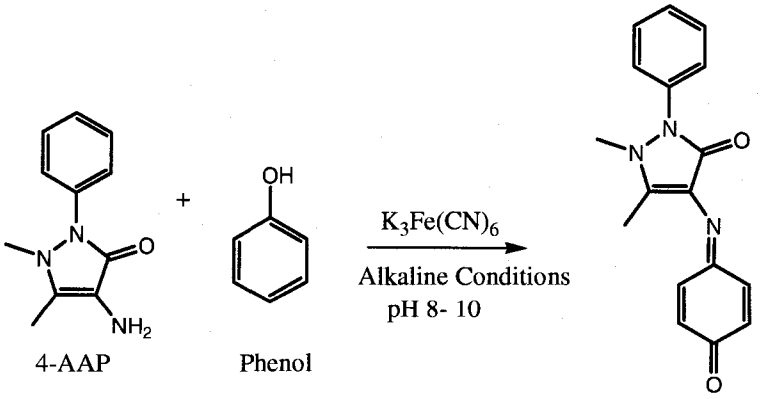
Yoshida K., Shigeoka T., Yamauchi F. **(1983)** Non-steady-state equilibrium model for the preliminary predicition of the fate of chemicals in the environment. ***Ecotoxicology and Environmental Safety*** 7:179-91.

Yu J., Taylor K.E., Zou H., Biswas N., Bewtra J.K **(1994)** Phenol conversion and dimeric intermediates in horseradish peroxidase catalyzed phenol removal from water. ***Environmental Scinece and Technology*** 28:2154-2160.

Zoppellaro, G., Sakurai T., Huang H.-W. **(2001)** A novel mixed valance form of *Rhus vericifera* laccase and its reaction with dioxygen to give a peroxide intermediate bound to the trinuclear center. ***Journal of Biochemistry*** 129:949-953

Appendix A: Phenol Colour Test

Colorimetric assay was initially designed to quantify phenolic compounds in wastewater and water samples. This assay is run in the pH range 8-10 in which 4-AAP performs an electrophilic attack on the para position of phenolic compounds forming a leuco compound which can be further oxidized to a quinone-type structure (Faust & Mikulewicz et al. 1967). Oxidative coupling can be blocked or altered by various groups in the para position of the benzene ring. CH_3-, C_2H_5- and C_6H_5- substituents, block 4-AAP coupling most likely due to lack of the ability of the substituent to be displaced or migrate to another position on the benzene ring. Cl-, Br-, CH_3O- and C_2H_5O- substituents alter oxidative coupling most-likely due to electrophilic displacement or, in the case of the amino group, to readily form and imine and hydrolyze. This explains the above-mentioned lack of chromophore formation for p-cresol. Standard curves were obtained by varying the concentration of a known phenolic compound and plotting against its absorbance at 510nm. Developing time was relatively short and stability of the product was confirmed up to 40 min following the reaction.



4-AAP Phenol Pink Chromophore

Scheme A.1: Phenol Colour Test Reaction. Colour intensity is proportional to phenol concentration.

Reagents

20 mM 4-AAP in 0.25 M $NaHCO_3$	83.4 mM $K_3Fe(CN)_6$ in 0.25 M $NaHCO_3$
- 0.2033 g of 4AAP	- 1.373 g of $K_3Fe(CN)_6$
- 12.5 mL of 1.0 M $NaHCO_3$	- 12.5 mL of 1.0 M $NaHCO_3$
- Dilute to 50 mL with DI H_2O	- Dilute up to 50 mL with DI H_2O

Procedure

1. Add sample volume 100-800 μL (dilute to 800 μL with DI H_2O if necessary)
2. Add 100 μL of 4-AAP reagent
3. Add 100 μL of $K_3Fe(CN)_6$ reagent (total volume of 1.0 mL)
4. Vortex and wait 8-15 min for colour development and measure absorbance at 510 nm.

Standard Curves for Phenol Colour Test

Standard curves made by plotting phenolic concentration (x-axis) *vs.* absorbance at 510 nm. According to Beer's Law, absorbance increases linearly with concentration over a narrow range and is reliable with values below 1.5. All standard curves based on a minimum of triplicate samples made independently of each other.

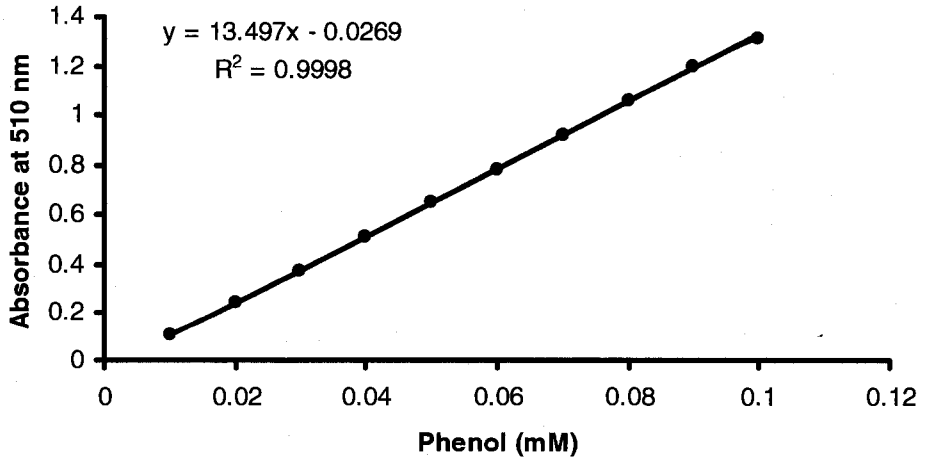


Figure A.1: Phenol standard curve with phenol colorimetric assay.

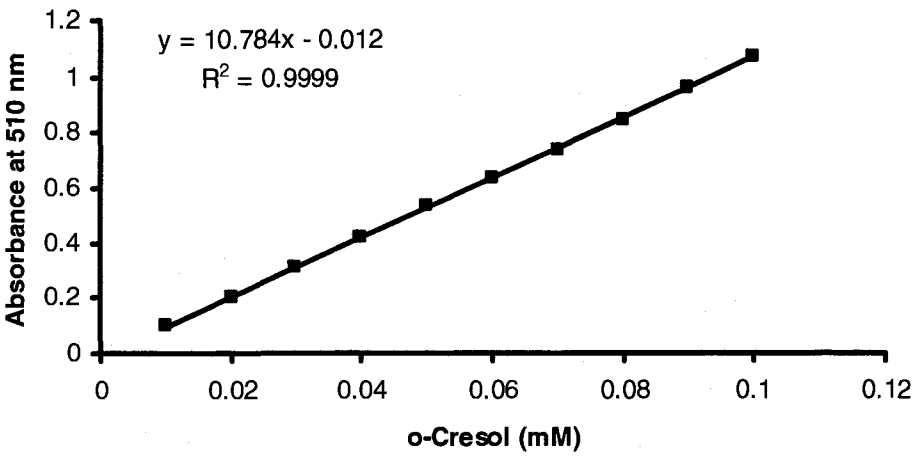


Figure A.2: o-Cresol standard Curve with phenol colorimetric assay.

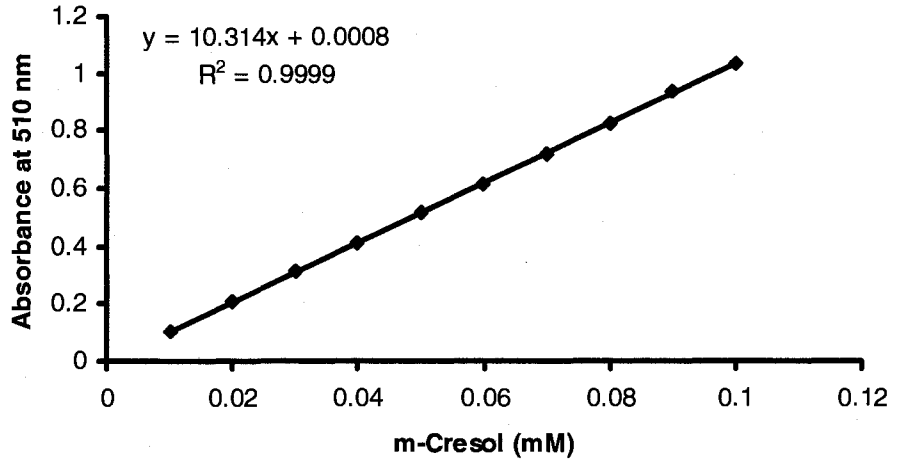


Figure A.3: m-Cresol standard curve with phenol colorimetric assay.

$A = \epsilon cl$

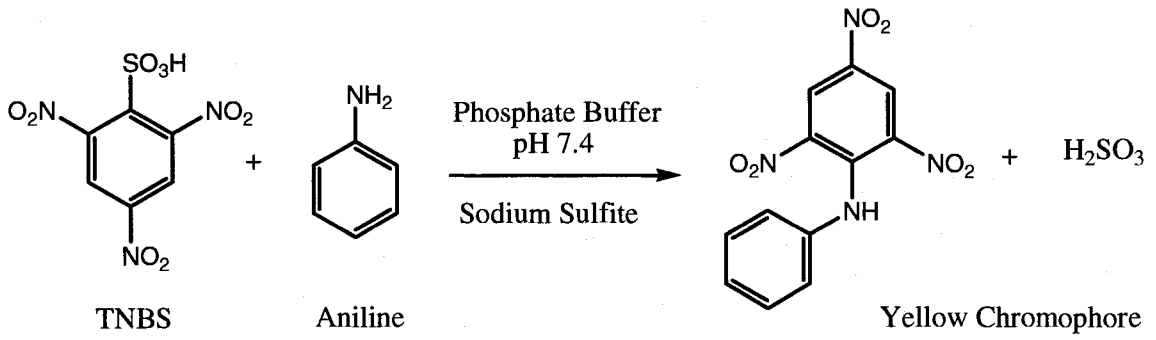
A = absorption, ϵ = molar absorbtivity extinction coefficient, c = concentration, p = path length

Linear form: $A = \epsilon c + b$, where b is the y – intercept

Table A.1: Summary of Phenol Colour Test Results		
Compound	**ϵ M^{-1} cm^{-1}**	**Developing Time (min)**
Phenol	13497	6
o-Cresol	10784	12
m-Cresol	10314	14
p-Cresol	N/A	N/A

Appendix B: TNBS Test

The TNBS colorimetric test method is a simple and accurate test for amines and anilines adapted from similar assays used for the determination of free amino acids. The reaction between amines and anilines occurs via a nucleophilic aromatic substitution with the formation of a N-trinitrophenyl product (Means et al. 1972). The addition of sodium sulfite allows for the formation of adduct with a longer wavelength that is stable enough for accurate quantification. Typical sulfite mono-adducts generate absorbances close to 420 nm. The intensity of the peak is dependent on sulfite concentration and it has been observed that at pH 7.4 this effect levels of with 2 mM sodium sulfite. Interference may be observed if aliphatic amines or strong anionic nucleophiles such as cyanide, azides or sulfide is present (Wang et al. 2005).



TNBS Aniline Yellow Chromophore

Scheme B.1: TNBS Colour Test. Generation of yellow chromophore is proportional to aniline concentration

Reagents:

10 mM TNBS	20 mM Na_2SO_3
0.0325 g diluted to 10 mL in DI H_2O (store in fridge at - 15°C, make fresh every few days)	0.252 g diluted in 100 mL in DI H_2O (make fresh daily)

Procedure

5. Add sample volume 100-700 µL (dilute to 700 µL with DI H_2O if necessary)
6. Add 100 µL of buffer (amines pH 9.2, anilines 7.4, due to difference in pKa's)
7. Add 100 µL of 10 mM TNBS
8. Add 100 µL of 20 mM Na_2SO_3
9. Vortex and wait for colour development and measure absorbance at 430nm.

Standard Curves for TNBS Test

Standard Curves made by plotting concentration of anilino compound (x-axis) versus absorbance (y-axis) at the appropriate wavelength. λ_{max} for the following substrates as well as developing time was determined by monitoring colour development in the wavelength range from 300 to 500 nm until colour development leveled off. All samples were stable for at least half an hour after the predetermined developing time (**Table B.1**). All standard curves were based on a minimum of triplicate samples made independently of each other.

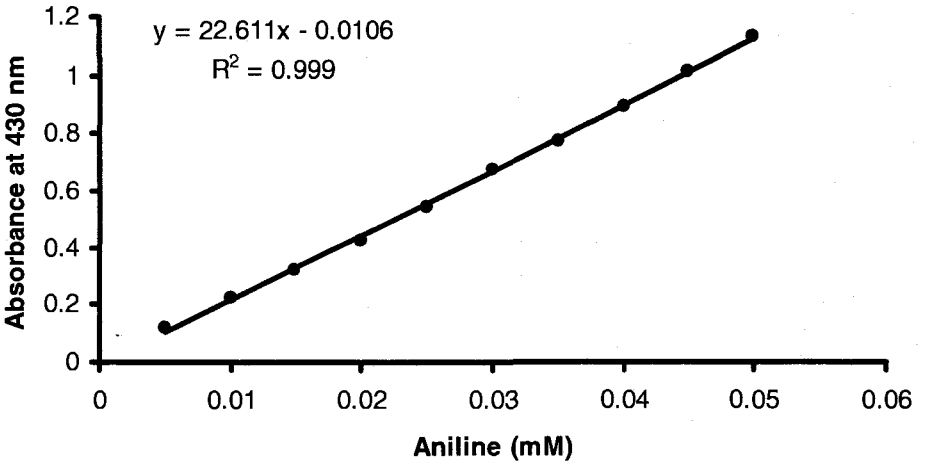


Figure B.1: Aniline standard curve with TNBS colorimetric assay.

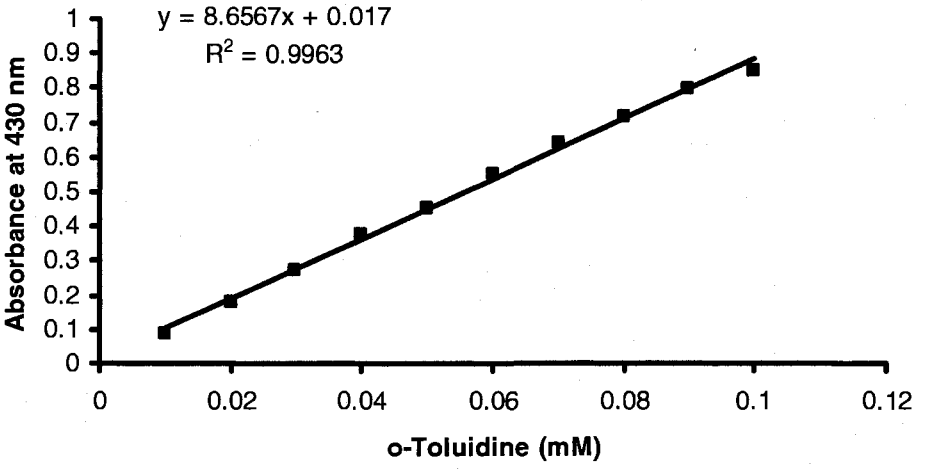


Figure B.2: o-Toluidine standard curve with TNBS colorimetric assay. Developing time of 200 minutes made this type of analysis impractical.

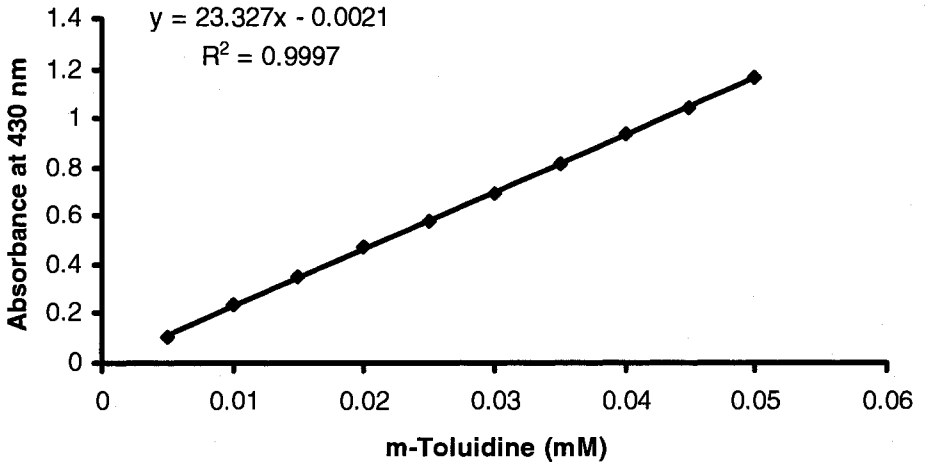


Figure B.3: m-Toluidine standard curve with TNBS colorimetric assay.

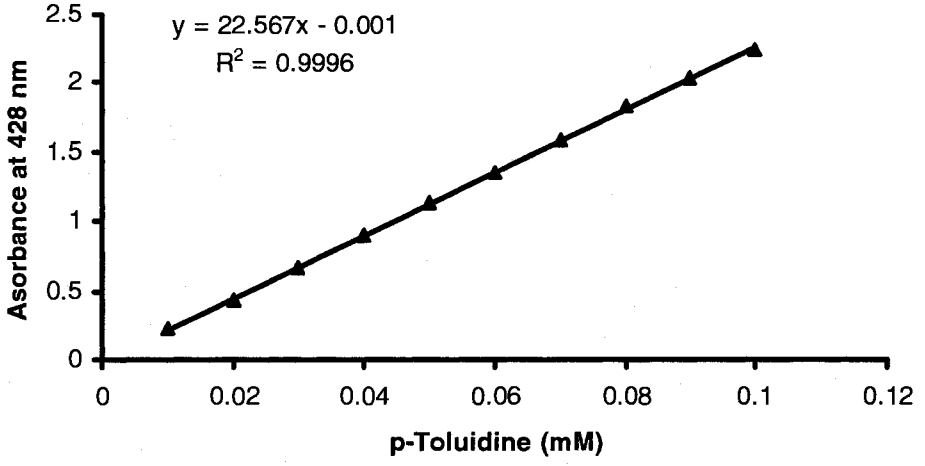


Figure B.4: p-Toluidine standard curve with TNBS colorimetric assay. Plot goes well above normal absorbancies based on Beers Law but because it remained linear the line was not truncated.

Summary of Results

Table B.1: Summery of TNBS Results		
Compound	**ϵ M^{-1} cm^{-1}**	**Developing Time (min)**
Aniline	22611	40
o-Toluidine	8658	200
m-Toluidine	23327	30
p-Toluidine	22567	20

Appendix C: Laccase Activity Assay

This method is based on the catalyzed oxidation of syringaldazine to its corresponding quinone detected by the increase in absorbance at 530 nm. One laccase unit is defined as the amount of enzyme required under the analytical conditions to convert 1 μmol of syringaldazine per minute at room temperature. Activity units at 5.5 are referred to as LACU and at 7.5 as LAMU (Felby 1998).

Reagents

1. 23 mM MES buffer with pH of 5.50 ± 0.05 (for SP 504)
   - 2.66 g of MES
   - 1.0 mL of 2 M NaOH
   - Diluted up to 500 mL in DI H_2O

   Or

   25 mM Tris buffer with pH of 7.50 ± 0.05 (for SP 805)
   - 12.5 mL of 1.0 M Tris (T1378)
   - 2.5 mL of 1.0 M maleic acid
   - Diluted up to 500 mL in DI H_2O

2. 0.38 mM syringaldazine solution
   - 6.8 mg syringaldazine
   - 25 mL of 96% ethanol (1.5 h mixing)
   - Diluted to 50 mL with DI H_2O
   - Store in the dark at 4°C (good for 4 days as long as its not mixed with any H_2O).

Procedure

Mix the reagents in the following order:

1. 850 μL of appropriate buffer.
2. 50 μL of syringaldazine solution
3. 100 μL of laccase sample

Total sample volume in cuvette should be 1.0 mL. Immediately after the addition of the laccase containing sample, the cuvette was shaken by inverting it three or five times in rapid succession and then placed in the spectrophotometer. Change in absorption at 530 nm was monitored by scanning every 15 s for 75 s.

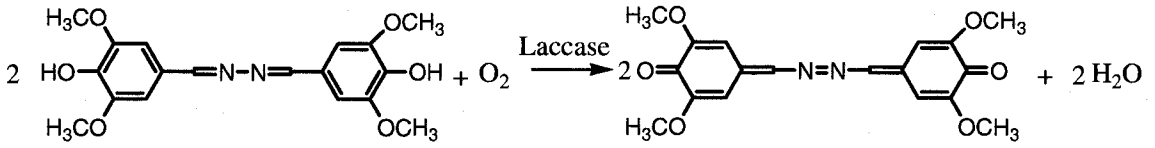
Calculation

LACU/mL or LAMU/mL is then calculated using the following formula:

Activity (LACU) = ((ΔA * 1 mL * 10-3)/ (0.065 * 0.1 mL)) * D = ΔA * 0.1538 * D

where: ΔA = Change in absorption per minute = (A_{75s} – A_{15s})
- Range of absorption should be 0.1 to 0.4 ΔA/min but trend is linear up to 1.0 ΔA/min.

1.0 mL is the sample volume in cuvette
0.065 = the μmolar absorption coefficient
D = dilution factor



Scheme C.1: Oxidation of syringaldazine to its corresponding quinone

Appendix D: HPLC Calibration Curves

HPLC calibration curves made under the same conditions as the analysis of substrate in batch reactors was done (See experimental methods). All standard curves were made with a minimum of triplicate samples made independent of each other.

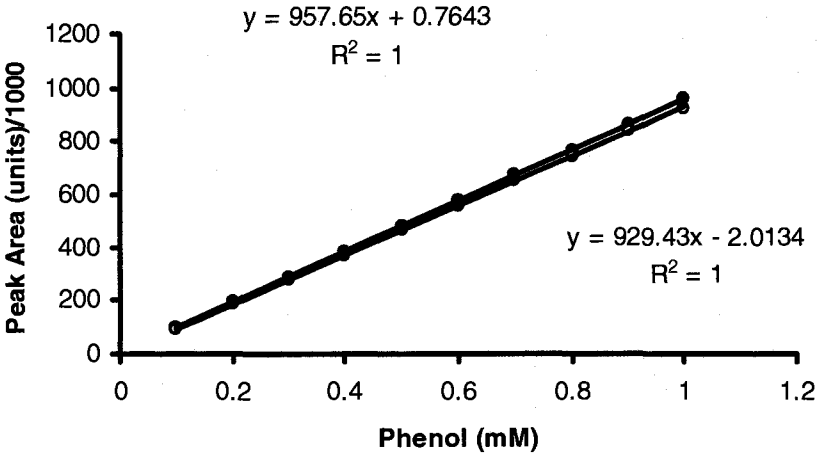


Figure D.1: Phenol HPLC standard curve with detector set at 270 nm. —•— Condition 1 was run with 60% acetonitrile and 40% 0.1% acetic acid (Retention time 2.34). —○— Condition 2 was run with 40% acetonitrile and 60% 0.1% acetic acid (Retention time 3.70).

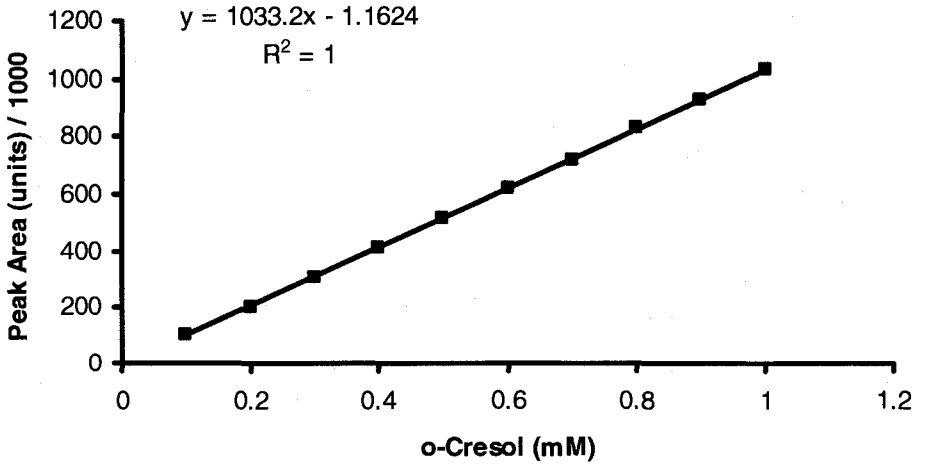


Figure D.2: o-Cresol HPLC standard curve; retention time ~ 2.8min.

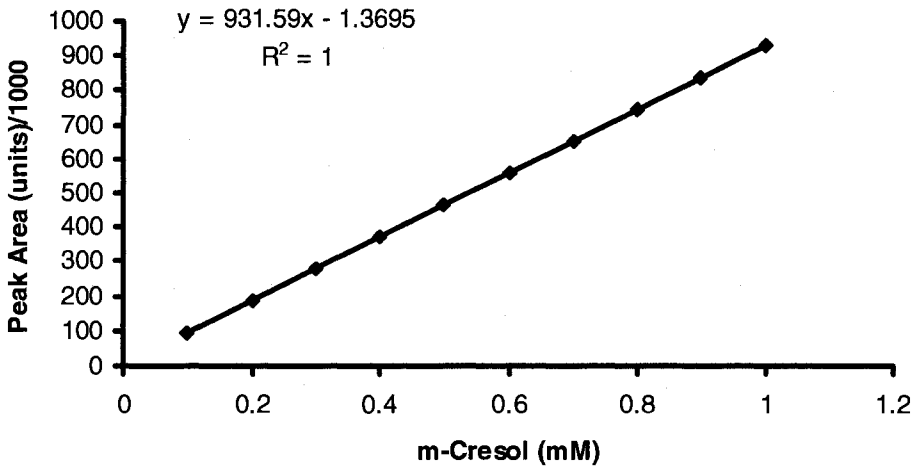


Figure D.3: m-Cresol HPLC standard curve; retention time ~ 2.6 min.

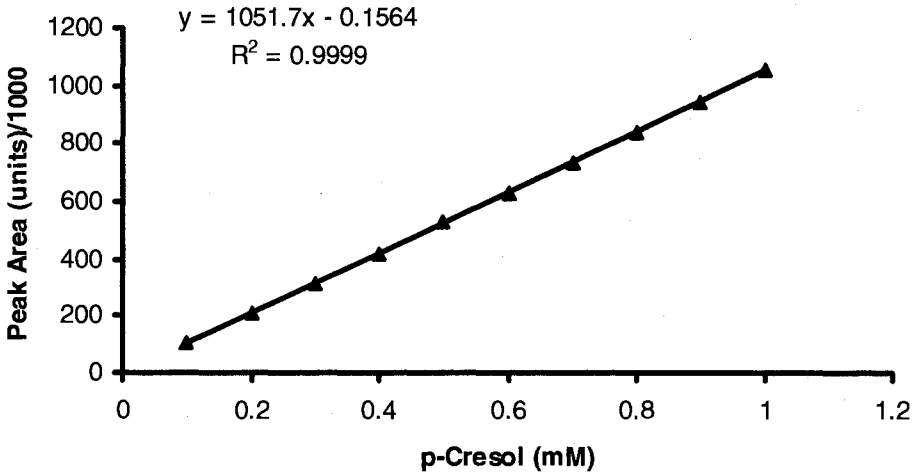


Figure D.4: p-Cresol HPLC standard curve; retention time ~ 2.6 min.

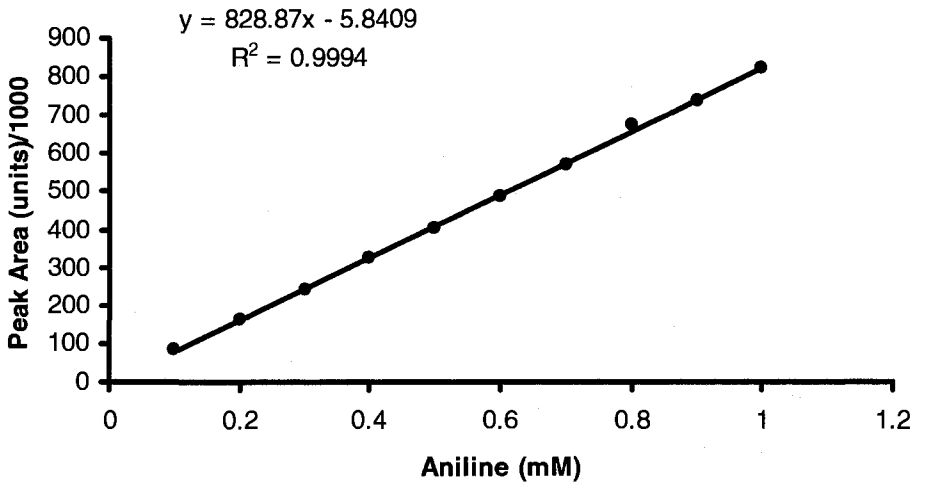


Figure D.5: Aniline HPLC standard curve; retention time ~ 3.2 min.

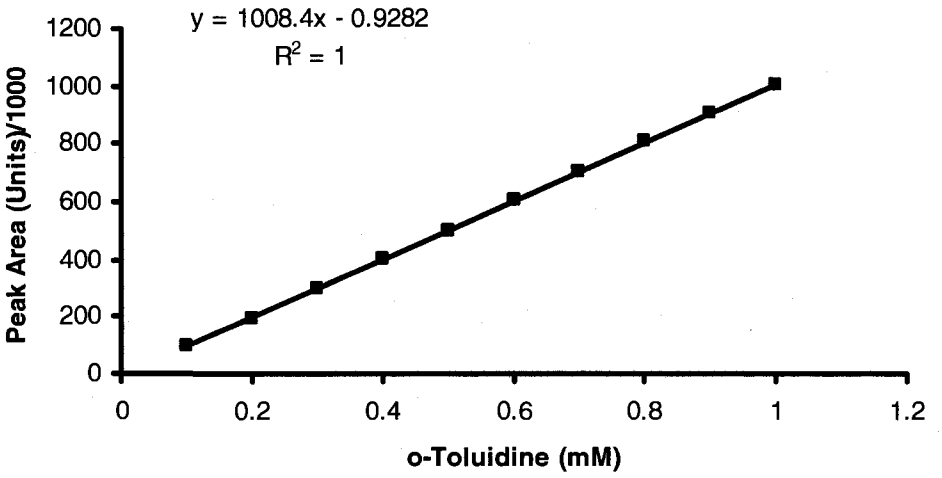


Figure D.6: o-Toluidine HPLC standard curve; retention time ~ 4.6 min.

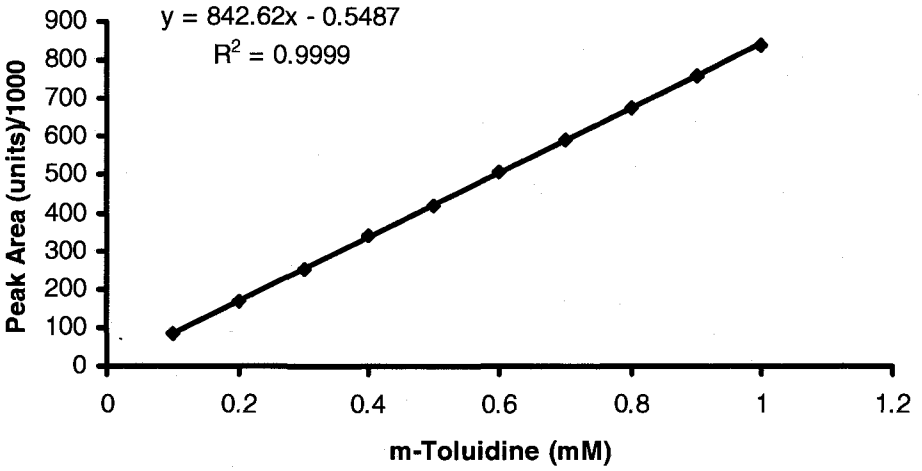


Figure D.7: m-Toluidine HPLC standard curve; retention time ~ 4.8 min.

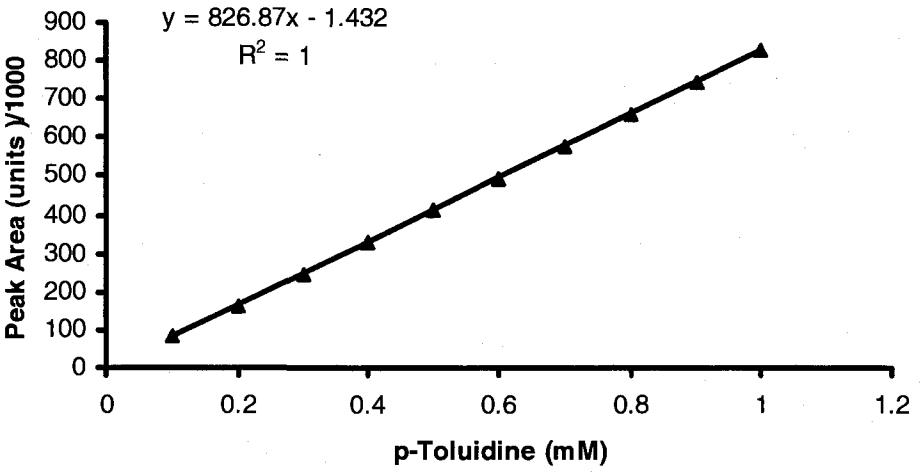


Figure D.8: p-Toluidine HPLC standard curve; ~ 4.8 min.

Appendix E: Direct Absorbance Standard Curves

Standard curves for aqueous solutions of substrates at or near the determined λ_{max}.

Summary can be found in **Table E.1** for comparison to literature values.

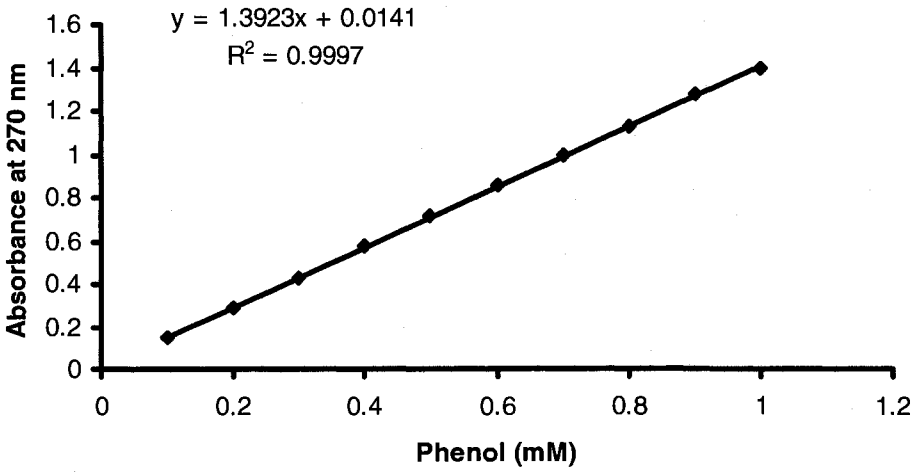


Figure E.1: Phenol standard curve by Direct Absorbance.

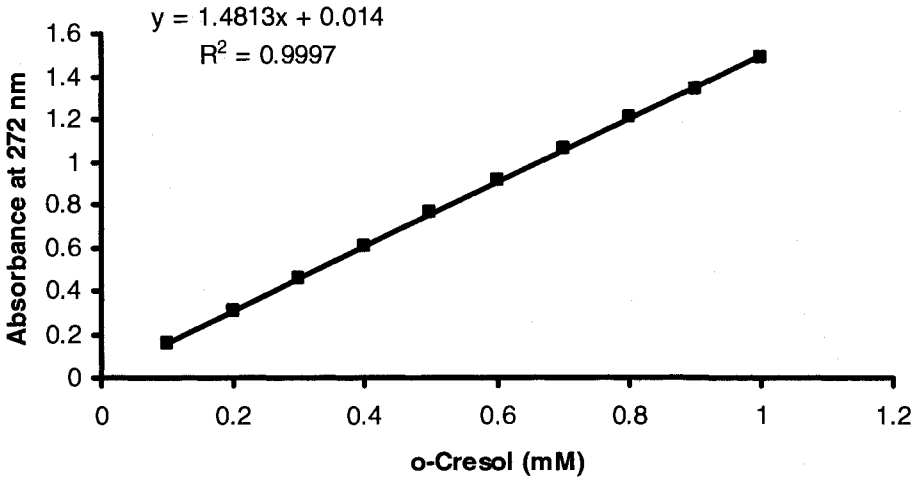


Figure E.2: o-Cresol standard curve by Direct Absorbance.

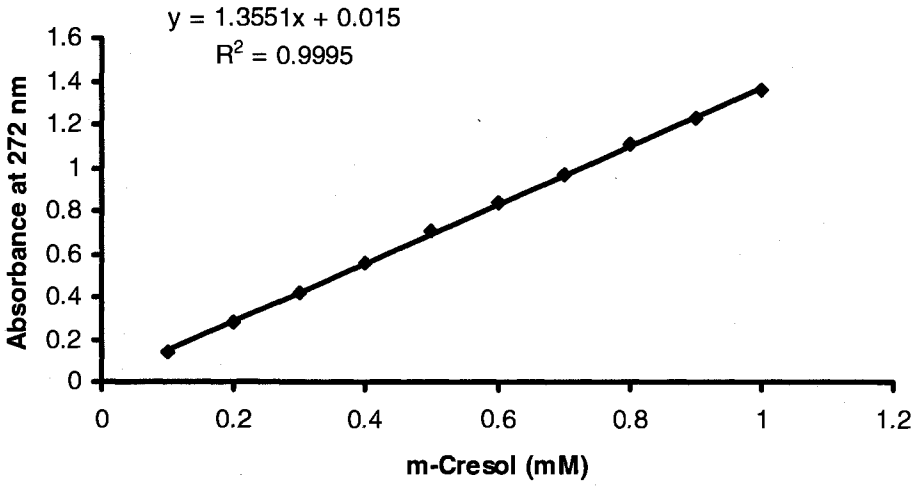


Figure E.3: m-Cresol standard curve by Direct Absorbance.

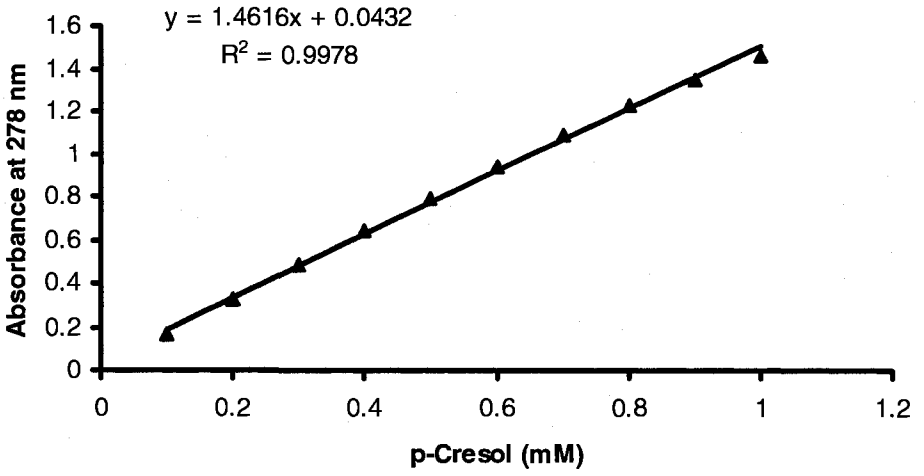


Figure E.4: p-Cresol standard curve by Direct Absorbance.

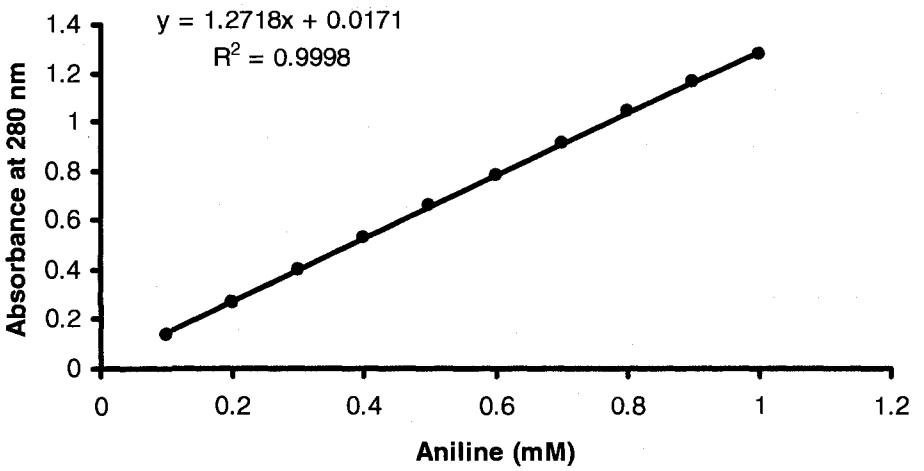


Figure E.5: Aniline standard curve by Direct Absorbance.

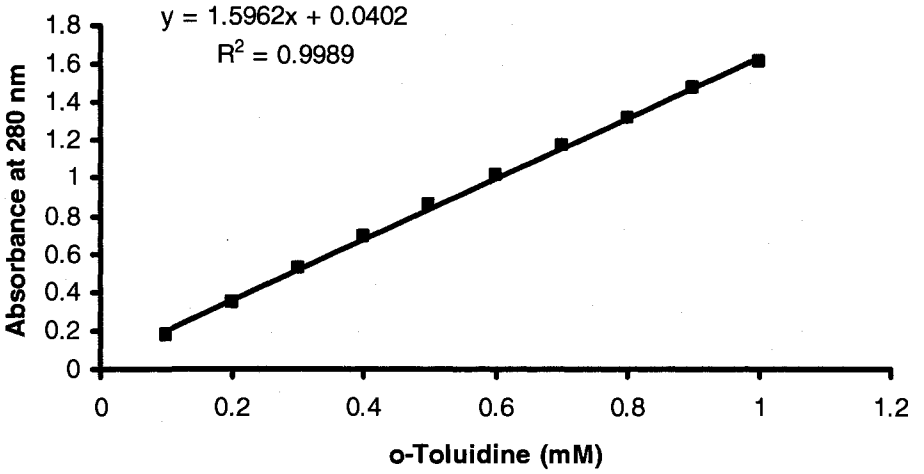


Figure E.6: o-Toluidine standard curve by Direct Absorbance.

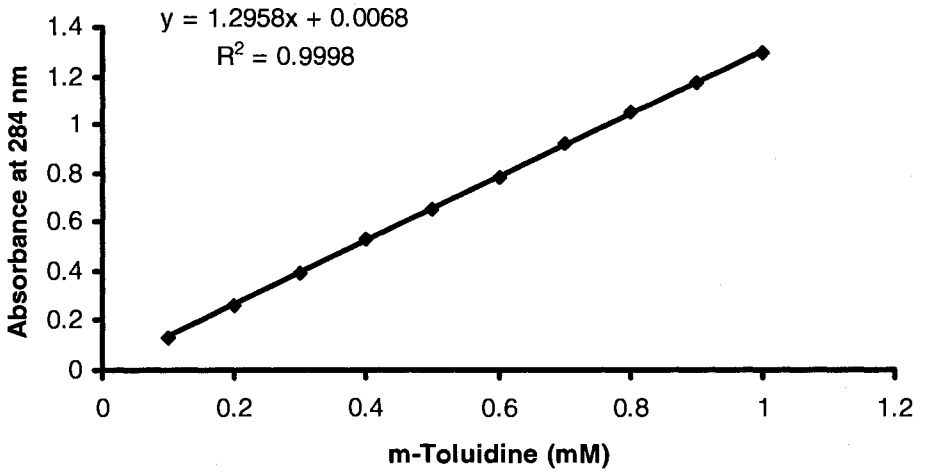


Figure E.7: m-Toluidine standard curve by Direct Absorbance.

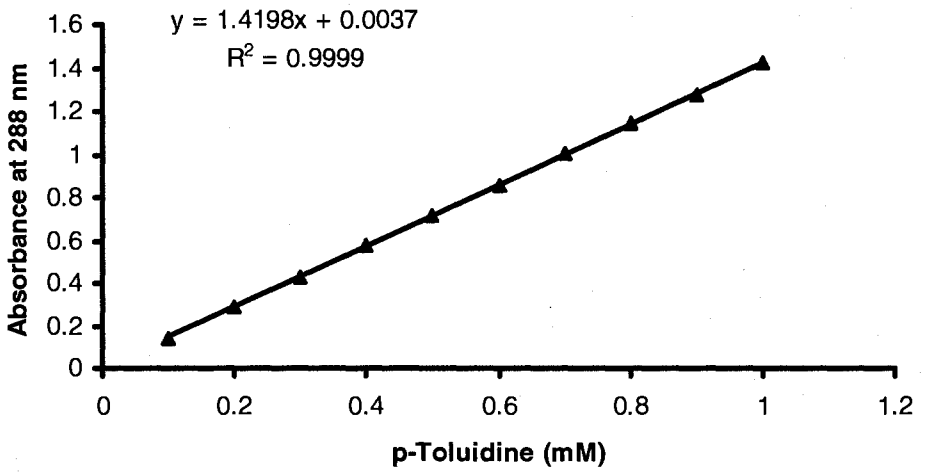


Figure E.8: p-Toluidine standard curve

Table E.1: Summary of Direct Absorbance		
Substrate	**ϵ M^{-1} cm^{-1}**	**Wavelength (nm)**
Phenol	1392	270
o-Cresol	1481	272
m-Cresol	1355	272
p-Cresol	1462	278
Aniline	1272	280
o-Toluidine	1596	280
m-Toluidine	1296	284
p-Toluidine	1420	288

All samples in aqueous solution.

Appendix F Kinetic Data

Table F.1: o-Cresol Initial Velocities With and Without PEG						
Initial o-Cresol (mM)	**No PEG**			**PEG**		
	Slope	**Intercept**	**R^2**	**Slope**	**Intercept**	**R^2**
0.2	0.0084	0.1952	0.9714	0.0068	0.1861	0.9918
0.4	0.0176	0.3931	0.987	0.0165	0.3787	0.9996
0.5	0.0222	0.4884	0.9867	0.0217	0.4781	0.9987
0.6	0.0268	0.5815	0.9805	0.0271	0.5706	0.9919
0.8	0.0313	0.7637	0.9769	0.037	0.7631	0.9903
1	0.0358	0.9524	0.9696	0.0435	0.944	0.9873
1.5	0.0431	1.4619	0.9381	0.0616	1.4726	0.9894
2	0.0475	1.9109	0.8958	0.0661	1.9195	0.9812
3	0.0526	2.8783	0.883	0.0732	2.8826	0.9568

Average of Duplicate Trials

Table F.2: p-Cresol Initial Velocities With and Without PEG						
Initial p-Cresol (mM)	**No PEG**			**PEG**		
	Slope	**Intercept**	**R^2**	**Slope**	**Intercept**	**R^2**
0.2	0.0039	0.1951	0.9959	0.0047	0.1944	0.9982
0.4	0.0084	0.4039	0.9834	0.0081	0.3936	0.9993
0.5	0.01707	0.4956	0.9904	0.0104	0.4958	0.9965
0.6	0.0119	0.6107	0.9904	0.0113	0.5941	0.9995
0.8	0.0139	0.811	0.9973	0.0152	0.7889	0.9975
1	0.0164	0.9849	0.9969	0.0176	0.9905	0.9931
1.5	0.021	1.4853	0.998	0.0233	1.4719	0.997
2	0.0246	1.97	0.9907	0.0246	1.9711	0.9903
2.5	0.0284	2.4704	0.9983	0.0273	2.4628	0.9962
3	0.0304	2.9347	0.9807	0.0292	2.9511	0.9997
4	0.0348	3.9074	0.9705	0.0311	3.9675	0.9963
5	0.038	5.0681	0.9821	0.0348	5.1165	0.9567
8	0.0446	8.028	0.9919	0.0357	8.1011	0.977
9.8	0.0415	9.8073	0.9721	0.0391	9.7867	0.996

Average of Duplicate Trials.

Table F.3: m-Cresol Initial Velocities			
Initial m-Cresol (mM)	**No PEG**		
	Slope	**Intercept**	**R^2**
0.5	0.0017	0.5241	0.76026
1.0	0.0047	1.0559	0.9504
1.5	0.0058	1.581	0.9806
2.0	0.0098	2.0503	0.9801
3.0	0.0144	3.0232	0.911
4.0	0.0162	3.9672	0.8624
5.0	0.0234	4.9483	0.7914
6.0	0.0178	5.941	0.7751
8.0	0.0274	7.8716	0.7561
10.0	0.0329	9.8987	0.8771
18.0	0.0485	17.889	0.747
24.0	0.0743	23.729	0.8273
28.0	0.0899	27.722	0.7106
36.0	0.0854	36.257	0.7376
40.0	0.1075	38.516	0.929
50.0	0.1531	46.869	0.8509
53.0	0.131	53.401	0.8999
60.0	0.1679	57.265	0.9992
80.0	0.1961	76.026	0.7845
90.0	0.2174	90.008	0.6308
109.0	0.2311	108.67	0.7341
126.0	0.2142	125.49	0.5899
142.0	0.3028	141.21	0.6444
145.0	0.2581	144.97	0.2545

Average of Duplicate Trials

VITA AUCTORIS

Name:	Aaron J. Steevensz
Placeof Birth:	Windsor, Ontario, Canada
Date of Birth:	July 16, 1980
Education:	PhD, Chemistry and Biochemistry University of Windsor Windsor, ON, Canada 2008
	B.Sc., [H] Chemistry and Biology Cum Laude (Double major) Graceland University 1 University Place, Lamoni, IA 50140 2003
Publications:	Steevensz A., Al-Ansari Mohammed Mousa, Taylor K.E. Bewtra J.K., Biswas N, (2008) Comparison of Soybean Peroxidase with Laccase in the Removal of Phenol in Synthetic and Refinery Wastewater Samples, Submitted to Journal of Chemical and Biotechnology
To be submitted:	Steevensz A., Taylor K.E., Biswas N., Bewtra J.K., (2008) Conversion of various aromatic phenols and anilines using a laccase from Trametes villosa and insights into the "PEG effect".
Abstracts:	Canadian Society for Chemistry (CSC) 89th Canadian Chemistry Conference, Halifax, Nova Scotia, May 27-31, 2006.Title: Enzymatic Treatment of Phenolic Compounds with the Aid of a Hydrophilic Synthetic Polymer, Polyethylene Glycol (PEG) – poster presentation
	41st Central Canadian Symposium on Water Research Quality (CAWQ), Burlington Ontario February 13-14, 2006. Title: Enzymatic Treatment of Cresols with Laccase SP504 and the Aid of a Hydrophilic Synthetic Polymer, Polyethylene Glycol (PEG) –oral presentation
Conferences Presentations:	9th Annual Chemistry and Biochemistry Research Conference, Concordia University Montreal, Quebec, November 24–25, 2006. Title: Removal of

Phenolics and Anilino Compounds using Laccase SP504 and the "PEG effect" –oral presentation

Interdisciplinary Graduate Student Research Symposium (IGSRS), McGill University, Montreal, Quebec, March 16-17, 2006.Title: Enzymatic Treatment of Phenolic Compounds with Laccase SP504 and the Aid of a Hydrophilic Synthetic Polymer, Polyethylene Glycol (PEG) –oral presentation

8th Annual Chemistry and Biochemistry Research Conference, Concordia University Montreal, Quebec, November18-19, 2005. Title: Enzymatic Treatment of Cresols Using Lacasse SP-504 with the aid of a Hydrophilic Synthetic Polymer, Poly(ethylene glycol) –oral presentation

7th Annual Chemistry and Biochemistry Research Conference, Concordia University Montreal, Quebec, November 19-20, 2004.Title: Enzymatic Treatment of Wastewater, Removal of Cresol With Laccase –poster presentation

Awards:

Ontario Graduate Scholarship (OGS) (September 2004 – May 2005) Under the Supervision of Dr. K. E. Taylor and Dr. N. Biswas, University of Windsor, Department of Chemistry and Biochemistry.

NAIA All American Scholar Athlete for Soccer, Graceland University, 2002, Coached by Ivan Joseph.